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PROGENITOR CELLS IN THE BRAINSTEM AND FILUM TERMINALE

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“Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.”

Sir Winston Churchill

To my family

ABSTRACT

Neurogenesis prevails in the adult mammalian CNS. New neurons are constitutively formed *in-vivo* in the subventricular zone (SVZ) of the lateral ventricle wall and in the subgranular zone of the dentate gyrus. However, other regions along the entire neuroaxis have been found to harbor cells with the ability to form neurospheres, self-renew and differentiate into neurons, oligodendrocytes and astrocytes.

Adult rodents were subjected to axotomies of the hypoglossal nerve (n.XII) followed by assessment of the endogenous neural progenitor cell (NPC) response and the fate of grafted adult eGFP SVZ NPCs in the brainstem. Sox2 expressing endogenous NPCs were abundant in the ependymal region of the central canal and 4th ventricle and activated after n.XII avulsion injury, which induced massive loss of motor neurons, but not after transection where the cell loss was minimal. Activation included NPC proliferation and subsequent migration to the hypoglossal nucleus (nucl.XII) where they expressed astrocytic markers. However, neurogenesis failed and no neuroprotective effect on motor neurons was observed. Therefore, a transplantation model was developed; SVZ NPCs were harvested from inbred eGFP transgenic animals and cells grafted to the nucl.XII of their wt siblings after n.XII injuries. The SVZ NPCs from transgenic animals were characterized *in-vitro* prior to grafting, and found to have same NPC characteristics as cells from wt animals. Upon transplantation after avulsion injury NPCs survived, differentiated into neurons, oligodendrocytes and astrocytes and integrated with the host circuitry. Grafted NPCs expressed VEGF and GPx1 that after avulsion exerted a neuroprotective effect on motor neurons. Moreover, we suggest that GPx1 expression contributed to the survival of transplanted cells in the nucl.XII post avulsion. This was in contrast to the results on grafting after transection since NPCs did not survive to the same extent and only differentiated into cells with astrocytic phenotype. Also, some of the cells continued to express Sox2. The cells did not integrate and no neuroprotective effects were found.

Differences in activation after the two types of axotomies were correlated to levels of motor neuron death in the nucl.XII, and hence differences in the microenvironment with regards also to levels of free radicals and inflammatory compounds.

The beneficial effects after grafting post avulsion initiated the development of an autologous transplantation model where the distribution of NPCs in the filum terminale was investigated. Sox2 and Mushashi-1 expressing cells in the human filum terminale were found, i.e. cells expressing NPC markers. The cells were abundant and when isolated and propagated *in-vitro* they formed neurospheres, proliferated and differentiated into neurons and glia. Clonal expansion indicated a relatively strong self-renewal capacity. The cells responded to the addition of PDGF-BB with an increase in neuronal cell numbers.

In summary, the findings suggest a repair strategy based on transplantation of adult NPCs in neural repair after severe motor nerve injuries. The filum terminale is a potential source of NPCs in future autologous cell therapies, even in human.

Key words: *brainstem, filum terminale, ependymal layer, subventricular zone, neural progenitor cells, nerve injury, motor neurons, transplantation, differentiation, integration*

LIST OF PUBLICATIONS

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

- I. Michael Fagerlund, Nasren Jaff, Alexandre I. Danilov, Inti Peredo, Lou Brundin and Mikael Svensson
Proliferation, migration and differentiation of ependymal region neural progenitor cells in the brainstem after hypoglossal nerve avulsion.
Restorative Neurology and Neuroscience 2011, 29(1):47-59
- II. Michael Fagerlund, Cynthia Pérez Estrada, Nasren Jaff, Mikael Svensson and Lou Brundin
Neural stem/progenitor cells transplanted to the hypoglossal nucleus integrates with the host CNS in adult rats and promotes motor neuron survival.
Cell Transplantation 2012, 21(4):739-747
- III. Michael Fagerlund, Cynthia Pérez Estrada, Nasren Jaff, Lou Brundin and Mikael Svensson
Integration differences of transplanted neural progenitors in two models of axotomy.
Manuscript
- IV. Lisa Arvidsson, Michael Fagerlund, Nasren Jaff, Amina Ossoinak, Katarina Jansson, Anders Hägerstrand, Clas B. Johansson, Lou Brundin and Mikael Svensson
Distribution and characterization of progenitor cells within the human filum terminale.
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ABBREVIATIONS

bFGF	Basic fibroblast growth factor
BMP	Bone Morphogenic Proteins
BDNF	Brain-derived neurotrophic factor
BrdU	5-Bromo-2'-deoxyuridine
CSC	Cancer stem cell
CD133	Cluster of differentiation 133
ChAT	Choline acetyltransferase
CNTF	Ciliary neurotrophic factor
CNS	Central nervous system
CSF	Cerebrospinal fluid
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
FGF	Fibroblast growth factor
FT	Filum terminale
GDNF	Glial cell line-derived factor
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
IGF	Insulin growth factor
JAK	Janus Kinase
iNOS	Inducible nitric oxide synthase
LIF	Leukemia inhibiting factor
MS	Multiple sclerosis
n.XII	Hypoglossal nerve
nNOS	Neuronal nitric oxide synthase
NGF	Neural growth factor
nucl.XII	Hypoglossal nucleus
NO	Nitric oxide
NOS	Nitric oxide synthase
NPC	Neural progenitor cell
PDGF	Platelet-derived growth factor
PNS	Peripheral nervous system
RNOS	Reactive nitric oxide species
ROS	Reactive oxygen species
RMS	Rostral migratory stream
SGZ	Subgranular zone
Shh	Sonic hedgehog

Sox2	Sex determining region of Y-chromosome (SRY)-related HMG-box 2
SVZ	Subventricular zone
Tg	Transgenic
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone

INTRODUCTION

Adult neurogenesis is today an accepted phenomenon of mammalian neurobiology. This, the firm establishment of the generation and development of new neurons in the adult central nervous system (CNS), was by The New York Times found to be the one finding that stood out as the most startling research result from the 1990-1999 “Decade of the Brain” (Blakeslee 2000).

In 1992 Brant A. Reynolds and Samuel Weiss published the first *in-vitro* evidence of adult neurogenesis when they induced cells isolated from the striatum of adult mice brain to proliferate and develop morphological and antigenic properties of neurons and astrocytes (Reynolds and Weiss 1992). Although this study was of uttermost importance as evidence for adult neurogenesis, some pioneering work was done already during the 1960’s by Joseph Altman which was later extended by Michael Kaplan and also by Fernando Nottebohm, which provided substantial evidence for the formation of new neurons in the adult (Altman 1962; Altman 1963; Altman and Das 1965; Kaplan and Hinds 1977; Goldman and Nottebohm 1983; Paton and Nottebohm 1984).

These findings contributed to the end and cessation of the “no neurogenesis dogma”. A dogma that prevailed for 100 years, posed by the founding fathers of neuroscience and exemplified by Santiago Ramón y Cajal when he in his 1913 *Estudios sobre la degeneración y regeneración del sistema nervioso* concluded that “*In adult centers, the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated*” (Ramón y Cajal 1928).

This has now changed and adult neurogenesis is established in rodent and non-human primate, findings show that it is also prevailed in the adult human CNS although this is to some extent debated (Eriksson et al. 1998; Curtis et al. 2007; Sanai et al. 2007; Sanai et al. 2011; Wang et al. 2011).

1 DEVELOPMENTAL NEUROGENESIS

The development of the nervous system commences when the mesoderm induces ectodermal cells to become lineage restricted as neural stem and progenitor cells, making the ectodermal cells specified as neural instead of non-neural epidermal cells – a process known as neural induction (Donkelaar et al. 2006). Hereby the neural plate, and subsequently the neural tube are formed. The neural tube is at first constituted by a

single layer of proliferating neuroepithelial cells that have the ability to give rise to all the neuronal and glial cell types in the developing CNS (Gotz and Huttner 2005). The neuroepithelial cells are highly polarized along their apical-basal axis, they express CD133, a transmembrane protein found in the apical plasma membrane, the intermediate filament Nestin and the Sex Determining Region of Y chromosome (SRY)-related HMG-box proteins 1, 2 and 3 (i.e. Sox1, Sox2 and Sox3) which act as transcription factors (Williams and Price 1995; Weigmann et al. 1997; Bylund et al. 2003; Graham et al. 2003; Gotz and Huttner 2005).

In the first phase of neural development these cells continuously divide, initially symmetrically, resulting in the formation of the primary proliferative and first germinative zone, the ventricular zone (VZ), which constitutes the wall of the primordial ventricle (Huttner and Brand 1997; Gotz 2003). This first proliferative phase and the length of it is considered to be the determinant for the given size of a specific brain region since this gives rise to a high or low number of precursor cells, and thus the onset of neurogenesis in time is influential for regional size.

At the onset of neurogenesis, the second phase, the neuroepithelial cells gives rise to the radial glia cells which are one of the earliest cell types to differentiate (Hartfuss et al. 2001; Fishell and Kriegstein 2003; Gotz and Huttner 2005). These cells extend from the luminal to the pial side in the developing CNS and functions both as precursor for neurons and glia and as guidance cells for newly generated neurons, leading them from the region of proliferation near the ventricular surface to their final position in the cortex.

The radial glia constitute the majority of precursors in the VZ and are distinguished from the neuroepithelial cells, as the name implies, by the development of glial characteristics. These include the appearance of glycogen granules, expression of glial fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST) and brain-lipid-binding protein (BLBP) (Hartfuss et al. 2001; Zecevic 2004). However, some of the neuroepithelial characteristics are maintained e.g. an apical surface and apical-basal polarity and the expression of Sox2, Nestin and CD133 (Hockfield and McKay 1985; Weigmann et al. 1997; Chenn et al. 1998; Hartfuss et al. 2001; Graham et al. 2003; Ellis et al. 2004). The radial glia are more fate restricted than the neuroepithelial cells and most of them give rise to a single cell type in the CNS i.e. neurons, oligodendrocytes or astrocytes (Gotz and Huttner 2005).

The symmetric cell division in the first phase is followed by asymmetric division, and a secondary proliferative layer is formed, the subventricular zone (SVZ) (Huttner and Brand 1997; Gotz and Huttner 2005). Basal progenitors, which originate from neuroepithelial and radial glia cells, contribute to the formation of this layer basal to the VZ. This secondary layer is present in the adult CNS as the SVZ of the lateral ventricle wall (Doetsch et al. 1997).

Neurogenesis is followed by the third phase, gliogenesis, where a distinctive expansion of the SVZ, which now contains many glial precursors, is seen. In the postnatal period the VZ is reduced in size in a progressive manner to eventually disappear and is in the adult replaced by the ependymal cell layer, although it is argued that a VZ is also contained within the adult germinal niche of the lateral ventricle wall (Ihrle and

Alvarez-Buylla 2011). The ependymal cells are also considered to be derived from radial glia (Spassky et al. 2005).

2 NEUROGENESIS IN THE ADULT

Adult neurogenesis is the production of new neurons in the adult CNS, which means that one, or a plethora, of neural stem, progenitor and/or precursor cells has to exist. The question is where, what, why and how?

2.1 Neurogenic Regions

Neurogenesis in the adult is not the same as neurogenesis during development. The information regarding position and patterning, conditions, starting points, the cells involved and the time course differ. However, knowledge about developmental neurogenesis is fundamental in order to comprehend the many aspects of adult neurogenesis and the links from the embryological and fetal cells, signals and events are obvious (Fishell and Kriegstein 2003; Gotz 2003; Gritti and Bonfanti 2007). For example, postnatal CNS development continues in the cerebellum and hippocampus although this has to be clearly set apart from adult neurogenesis.

In the adult there are two regions where neural stem or progenitor cells are present in a milieu, or niche, which allows for new neurons to be formed; the SVZ in the lateral ventricle wall and the subgranular zone (SGZ) in the dentate gyrus in the hippocampus (Riquelme et al. 2008; Ihrie and Alvarez-Buylla 2011; Ming and Song 2011). These are the neurogenic niches in the adult brain where neurogenesis prevails under normal physiologic conditions.

Cells from these two neurogenic regions can be isolated, form neurospheres, self-renew, express phenotypic markers for stem or progenitor cells and differentiate into neurons, oligodendrocytes and astrocytes. They have been studied extensively in *in-vitro* models, originally described by Reynolds and Weiss and further characterized by others (Reynolds and Weiss 1992; Morshead et al. 1994; Reynolds and Weiss 1996; Gritti et al. 1999). These *in-vitro* characteristics have been the basis and golden standard for research on adult neurogenesis in general, and more specifically research on identifying the true, if there is one, stem cell of adult neurogenesis. Thus, it is important to stress that culture conditions can affect cell characteristics and there are several reports on cells which appear to be neural stem or progenitor cells in culture, but *in-vivo* fail to fulfill the criteria (Goritz and Frisen 2012). On the other hand, the failure of neuronal differentiation does not per se mean that a response is of no use since the cell needed in a particular instance could very well be, for example, an astrocyte.

Subventricular zone

The SVZ in the wall of the lateral ventricle is the largest region of neurogenesis in the adult mammalian brain. Cells from here migrate from their position in the SVZ, via the anatomical structure known as the rostral migratory stream (RMS), in a migration called chain migration to the olfactory bulb where they generate inhibitory olfactory bulb interneurons (Doetsch and Alvarez-Buylla 1996; Lois et al. 1996; Curtis et al. 2007).

The architecture of the SVZ in the adult was originally described by Arturo Alvarez-Buylla and his group in 1997, and consists of four major cell types; 1) the slowly dividing GFAP-positive SVZ-astrocyte (*Type B cell*) which is considered to function as the primary neural stem/progenitor cell in the adult, 2) the rapidly dividing transit-amplifying cell (*Type C cell*) which functions as a precursor cell, this cell is also sometimes referred to as the intermediate precursor cell, 3) the migrating neuroblast (*Type A cell*) which is derived from the type C cell and migrates along the RMS to the olfactory bulb for terminal differentiation and 4) the ependymal cell (Doetsch et al. 1997).

The complexity of this region extends beyond these cells since several other components and factors contribute to, and are significant for, the features and function of neurogenesis in this neurogenic niche.

These other constituents involve microglia, the vasculature including endothelial cells, fibroblasts, cerebrospinal fluid (CSF), extracellular matrix proteins, gap-junctions, basal lamina, extrinsic signaling pathways e.g. growth factors, intracellular acting molecules e.g. transcription factors and neurotransmitters e.g. GABA signaling (Coskun et al. 2008; Riquelme et al. 2008; Ihrie and Alvarez-Buylla 2011; Ekdahl 2012). Furthermore, this niche has been found to be heterogeneous as the stem/progenitor cells in different locations give rise to distinct progeny in the form of different types of neurons.

As mentioned above the SVZ is a remnant of the embryonic SVZ and the multiciliated ependymal cells replaces the psuedostratified epithelium of the VZ turning it into a simple columnar epithelium lining the ventricle wall. During the years there has been a discussion about the identity of the primary neural stem/progenitor cell in the adult SVZ and many papers have dealt with the quest of presenting evidence enough for a general acceptance of its nature. Jonas Friséns group demonstrated that this cell would be the ependymal cell, this was however argued by others and Arturo Alvarez-Buylla and colleagues in his lab presented evidence showing that this cell was instead the type B astrocyte-like cell of the SVZ (Doetsch et al. 1999; Johansson et al. 1999). The latter has been supported in additional studies, and the GFAP-positive type B cell is more or less considered to be the “true” stem/progenitor cell in this neurogenic region of the adult nervous system. The type B cells have been found to have apical processes that penetrates the ependymal layer which makes this cell, as well as the ependymal cell, to be in contact with the CSF (Doetsch et al. 2002; Ihrie and Alvarez-Buylla 2011; Kokovay et al. 2012). Furthermore, it was suggested that the ependymal cells are born in the embryonic and early postnatal brain and after differentiation are postmitotic, and do not contribute to neurogenesis during normal physiologic conditions (Spassky et al.

2005; Carlen et al. 2009). Although the, also radial glia derived, ependymal cell is proposed to possess neural stem cell characteristics in the adult (Coskun et al. 2008). Whether neurogenesis is continuously ongoing in the SVZ also in the adult human CNS is debated and it appears that the SVZ of the adult human brain differs from the SVZ of the non-human mammalian brain, however there are some evidence favoring neurogenesis also in man (Curtis et al. 2007; Sanai et al. 2007; Sanai et al. 2011; Wang et al. 2011).

Dentate gyrus

The second neurogenic region of the adult CNS is the dentate gyrus of the hippocampus. Also here the link to development is present as a radial glia-like cell in the SGZ is the putative stem/progenitor cell of this niche. From these quiescent cells intermediate precursors and subsequently neuroblasts are formed which, together with immature neurons, migrate to the inner granule cell layer where they differentiate into excitatory dentate granule cells that are generated and integrated into the existing circuitry throughout life (Eriksson et al. 1998; Filippov et al. 2003; Ming and Song 2011; Encinas and Sierra 2012).

In this part of our nervous system declarative information is processed and consolidated by relating items in space and time, thus enabling memory to be episodic and neurogenesis in the SGZ has, interestingly enough, been shown to be affected by factors such as physical exercise, enrichment of the milieu surrounding the individual and stress (Gould et al. 1997; Kempermann et al. 1998; van Praag et al. 1999; Aimone et al. 2011; Ming and Song 2011).

Regarding this neurogenic region adult neurogenesis can be considered established also in the human, although it appears to be sparse (Eriksson et al. 1998; Goritz and Frisen 2012).

Since this region, although highly dynamic and interesting, is not the focus for this study the interested reader is referred to the literature cited for further reading.

2.2 Non-Neurogenic Regions

In most regions of the adult CNS neurogenesis does not occur. The neurogenic regions are defined by a germinative matrix where neurogenesis persist throughout life, and during normal physiological conditions the SVZ and the SGZ are the only parts of the adult CNS where this occurs. Nonetheless, other regions along the entire neuroaxis have been found to harbor cells with the ability to form neurospheres, self-renew and differentiate into neurons, oligodendrocytes and astrocytes.

Ependymal region of the 4th ventricle and central canal

The ciliated columnar ependymal cell originates from the radial glia cell and forms an ependyma i.e. epithelial monolayer lining the surface of the CSF containing ventricles

and the central canal, and plays important functions in adult neurogenesis as part of the neurogenic niche in the SVZ, e.g. for integrity of the niche and in pathway signaling (Sturrock and Smart 1980; Spassky et al. 2005; Gritti and Bonfanti 2007; Barnabe-Heider et al. 2008; Coskun et al. 2008; Morrens et al. 2012).

The ependymal cell is considered quiescent and seem not to contribute to neurogenesis under normal physiological conditions in the SVZ niche, although others suggest different (Spassky et al. 2005; Coskun et al. 2008; Carlen et al. 2009; Ihrie and Alvarez-Buylla 2011). That is, it does not contribute by means of vast proliferation and differentiation.

The ependyma is demonstrated to be replaced in models of ependymal depletion which would indicate that ependymal cells are not post-mitotic and do divide in the adult, however findings show that this is accomplished by dividing SVZ astrocytes which incorporate within the ependyma where they develop characteristics of ependymal cells (Spassky et al. 2005; Luo et al. 2006; Luo et al. 2008; Nomura et al. 2010).

Interestingly, it is also found that ependymal cells can convert to astrocytes and that the majority of the SVZ astrocytes that are incorporated in the ependymal layer for ependyma maintenance derive from ependymal cells, an interconversion which seems to occur without cell division (Nomura et al. 2010).

Thus, the quiescent ependymal cell is indeed plastic and of the uttermost importance for SVZ neurogenic niche maintenance and function, furthermore it is shown to give rise to both neuroblasts and astrocytes in the forebrain during pathological conditions e.g. stroke (Carlen et al. 2009). Importantly, the ependymal cell in the central canal have been found to proliferate also in the uninjured setting, however also in this part of the CNS they are considered a quiescent population of very slowly dividing cells which self-duplicates only in order to maintain their populations in the ependymal layer (Hamilton et al. 2009; Barnabe-Heider et al. 2010).

But more important, ependymal cells of the fourth ventricle and central canal region of the brainstem and spinal cord have been reported to proliferate, migrate and differentiate upon spinal cord injury and inflammatory disease (Johansson et al. 1999; Brundin et al. 2003; Mothe and Tator 2005; Danilov et al. 2006; Meletis et al. 2008; Cizkova et al. 2009; Barnabe-Heider et al. 2010; Foret et al. 2010). The ependymal cell of this region is also shown to respond to non-pathological physiological stimulation (Cizkova et al. 2009; Foret et al. 2010).

Thus, although maybe not considered to be the cell from which all adult neurogenesis originates, the ependymal cell has been shown to possess neural stem or progenitor cell abilities *in-vitro*, and also *in-vivo* following injury and disease.

Filum terminale

Since cells around the central canal possess multipotent progenitor cell abilities, interest was raised in the filum terminale (FT) for investigations regarding adult neurogenesis and potential use in regenerative medicine.

The FT is a structure that extends from the conus medullaris to the coccyx. It has generally been considered to be a fibrous structure with longitudinal arrangement of

collagen bundles and large amounts of elastic fibers giving it considerable elastic properties, with its main clinical significance in the stretch induced functional disorder; tethered cord syndrome (Fontes et al. 2006; Liu et al. 2011). However, findings also suggested that the FT was a continuation of the spinal cord with an ependyma-lined central canal, neurons and glia cells and the question whether the small sized neurons found could represent neurons in an early phase was raised (Choi et al. 1992; Rethelyi et al. 2004; Fontes et al. 2006). The development of the FT involves the process of secondary neurulation where amongst other events the neuroepithelial cells of the primitive spinal cord subsequently are substituted after a sequence of aggregation and apoptosis (Nievelstein et al. 1993; Salbacak et al. 2000).

The FT is thus a much more complex structure than a fibrous streak and it has been shown that the adult human FT contains progenitor cells that *in-vitro* self-renew, proliferate to form neurospheres and express phenotypic markers for neurons, oligodendrocytes and astrocytes and also develop the electrophysiological profile of neurons and glia (Varghese et al. 2009).

Parenchyma of the spinal cord

As adult spinal cord cells in culture responded to epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) by proliferation and differentiation into neurons and glia, it was concluded that neural stem/progenitor cells were present also within here (Weiss et al. 1996; Shihabuddin et al. 1997). Although not fully characterized these parenchyma cells have been reported also *in-vivo* and found to express markers associated with neural stem or progenitor cells and to respond to injury and disease (Horner et al. 2000; Azari et al. 2005; Ohori et al. 2006). Thus, in addition to cells in the ependymal layer, stem/progenitor cells are present also in the parenchyma.

Although not being the focus or examined in detail in the work of this study, knowledge of their existence is of importance when investigating neural stem/progenitor cells in the brainstem. However, these cells seem not to be as proliferative and multipotent as the ependymal cell from the central canal (Martens et al. 2002; Ohori et al. 2006; Meletis et al. 2008).

The spinal cord environment is in general gliogenic i.e. it is inhibitory to neuronal differentiation and the regeneration of axons in the CNS is hindered by the constituents in this particular niche, thus neural stem/progenitor cells are found to give rise mainly to glia and only limited numbers of neurons after both transplantation and endogenous activation (Cao et al. 2001; Ohori et al. 2006; Parr et al. 2007). This gliogenic feature of the spinal cord is supported by transplantation experiments where cells transplanted to the hippocampus generated neurons whereas the same cells grafted to the spinal cord generated cells of glial nature (Shihabuddin et al. 2000).

2.3 Identifying the Progenitor Cell

The use of the term stem cell in adult neurogenesis is somewhat confusing and controversial. Therefore, it is not overwhelmingly hard to understand why researchers

within the field compares the difficulties on defining an adult neural stem cell to the frequently quoted remark made by U.S. Supreme Court Justice Byron White on something completely different: “It is hard to define, but I know it when I see it” (Morrison et al. 1997). When I first entered the world of adult neurogenesis and commenced my scientific work this was kind of how I experienced it. I intuitively knew what an adult neural stem cell was. Or, to be honest, I thought I knew. Not to be forgotten is that many conclusions regarding the identity, and whole nature, of the neural stem or progenitor cell have to be drawn from *in-vitro* experiments and *in-vitro* data. This means that the identification of features of a cell involved in adult neurogenesis are, at least to some extent, dependant on the methods applied.

Phenotypic markers

In order to conduct research on adult neurogenesis it is crucial to identify the adult neural stem or progenitor cell.

For this a number of markers have been found to be expressed by such a cell, of which many obviously are found in stem, progenitor and precursor cells during development as previously discussed. Two of these markers, which are used when conducting research on adult neurogenesis, are the type VI intermediate filament Nestin and the transcription factor Sox2 (Doetsch et al. 1997; Filippov et al. 2003; Ellis et al. 2004; Komitova and Eriksson 2004; Mothe and Tator 2005; Baer et al. 2007). Other markers used for identifying adult neural stem/progenitor cells are the neural RNA-binding protein Musashi-1, the transmembrane protein Notch1 which signaling pathway is important in maintaining a stem/progenitor cell state and inhibiting neuronal differentiation, the adhesion and signaling molecule vascular endothelial adhesion molecule-1 (VCAM1), identified as a key molecule responsible for maintaining the neurogenic niche and GFAP, which of course is not restricted to stem or progenitors, but is also one of the key features for astrocytes (Doetsch et al. 1997; Kaneko et al. 2000; Stump et al. 2002; Kokovay et al. 2012).

Importantly, no single marker stands out as the specific marker for identifying adult neural stem or progenitor cells. For example Nestin is for sure expressed by these cells, but also by reactive astrocytes and cannot be used as an exclusive marker. Instead a combination of expression of various markers has to be used, which also might reflect the heterogenic character of this cell and cell niche.

Defining the neural progenitor cell

By strict definition a stem cell should be totipotent, i.e. it should not only have the capability to differentiate into all the specialized cells in an organism, but also be able to give rise to cells and tissue outside of the embryo, however associated with the embryo (i.e. the trophoblast). This cell, the zygote, the mother of all stem cells, is a true stem cell and can give rise to a complete new organism.

When working on adult neurogenesis the terms stem, progenitor and precursor cells are all being used, mostly for describing different cells which differs with regards to characteristics and “stemness”, but not always. Most neuroscientists generally accept that the term adult neural stem cell include cells that are “only” pluri or multipotent and that the term progenitor cell might be used equipotent. It is important to stress that the cell must fulfill the basic criteria including ability to self-renew and differentiate to one or more types of specialized daughter cells, this could be both by symmetric and asymmetric cell division. More strict developmental definitions gives that this cell should be a cell that self-renews during the lifetime of the organism, to exclude transient precursors, and that the term progenitor cell should be used to describe a cell with limited self-renewal and with somewhat limited stem cell properties, for reviews see (Weissman et al. 2001; Seaberg and van der Kooy 2003).

In this thesis the term neural progenitor cell (NPC) will be used. By this I refer to a cell that, within the adult CNS, has the ability to self-renew and give rise to neurons, oligodendrocytes and astrocytes. Whether this cell has the ability to unlimited self-renewal and the potential to generate, if not all, but at least multiple cell-lineages within the organism is not stated – hence the term progenitor cell rather than stem cell. Neither do I claim to have identified the exact identity, nature nor feature of a cell from which adult neurogenesis originates. This is a quest prevailed by many others and for which the answer is not yet given. However, when working in the field of adult neurogenesis and drawing conclusions from experimental work, this heterogeneity is important to bear in mind.

3 PERIPHERAL NERVE INJURY

The peripheral nervous system (PNS) is composed of the spinal nerves and roots, the peripheral nerves, autonomic fibers in the periphery and also the cranial nerves. Within the scope of the investigations and results presented herein, only the motor peripheral nervous system will be discussed.

The motor neurons in the PNS (i.e. the lower motor neurons) have their cell bodies located in the spinal cord or in the brainstem in the CNS. The axons of these motor neurons initially run a short distance within the CNS passing the interface to the PNS to continue in the peripheral nerve to eventually end up in its target organ, the muscle.

3.1 Retrograde and Anterograde Degeneration

An injury to axons of motor neurons in the adult PNS induces a series of structural and metabolic alterations in the injured neuronal cell bodies usually referred to as the retrograde “axon reaction”. This was originally defined based on morphological characteristics including swelling of the soma, decentralization of the perikarya and dispersal of Nissl substance “chromatolysis”. However, retrograde changes that occur in the CNS following peripheral axotomy includes also a number of structural changes in the immediate vicinity to the affected motor neurons, including the glial cell

response, “gliosis” and detachment of synapses “synaptic stripping”. These retrograde changes in the CNS are paralleled by peripheral reorganization including disintegration of the disconnected distal portion of the axon, the anterograde “Wallerian” degeneration, Schwann cell proliferation followed by sprouting and regeneration. For review see (Lieberman 1974; Aldskogius 1993).

The “Axon Reaction”

Neuronal cell death

The outcome following axotomy can be grouped in three principally different end results for the neurons; I) complete recovery, II) atrophy with partial recovery or III) degeneration and death. However, the degree of cell death has been suggested to be overestimated since the atrophy of these cells can be substantial and long lasting, and thus hard to find in the tissue in cell counting experiments (Kwon et al. 2002). Findings, initially from axotomized rubrospinal neurons and later also in motor neurons in the facial nucleus after facial nerve axotomy, showed that atrophied neuron perikarya recovered to more normal volumes following a second axotomy (Kwon et al. 2002; McPhail et al. 2004). This discrepancy, and perhaps overestimation of neuronal cell death, indicates that even after some time there are cells to be rescued, which were thought to be lost, i.e. treatment also in the chronic state after injury could be worthwhile. In addition, when assessing cell loss it is found that neuronal loss in for example the hypoglossal nucleus is not uniform as the loss is more prominent at caudal levels (Yu 1988). These results should, at least, be considered when evaluating results.

The exact mechanisms leading to neuronal death are not fully understood, but it appears that trophic support e.g. provided from the periphery by proliferating Schwann cells and target contact is of fundamental importance (Carlstedt et al. 1989; Koliatsos et al. 1994; Li and Raisman 1994; Wu et al. 1994; Novikov et al. 1997), for review see (Chu and Wu 2009).

How motor neurons die is also to some extent unclear but it is more or less concluded to be by both programmed, apoptotic, and by means of necrotic death (Li et al. 1998; Martin et al. 1999; Park et al. 2007).

Detachment of synapses

“Synaptic stripping”, coined by Kreutzberg, seems to be an important endogenous neuroprotective mechanism, by providing protection from the metabolic stress of excitotoxic transmission that may be harmful to the axotomized neuron (Blinzinger and Kreutzberg 1968). The removal of synapses begins early after the axotomy which is well documented in several publications by e.g. counting their numbers on the cell surface of affected neurons (Svensson et al. 1991; Svensson and Aldskogius 1993). Detachment of synapses are paralleled by a reduction in size of the dendritic tree related to down regulation of the microtubule associated protein MAP2 in the dendrites, loss of axon collaterals and a reduction in the diameter of the proximal part of the axon (Risling et al. 1983; Havton and Kellerth 1990; Svensson and Aldskogius 1992).

Microglia

The glial responses in the CNS that follows a peripheral nerve injury includes proliferation and migration of reactive microglial cells that early in the process become located near the cell surface (perineuronal) of affected neurons (Svensson and Aldskogius 1993; Aldskogius 2001; Moran and Graeber 2004; Graeber and Streit 2010). The microglial response seem to be an important “player” in a cascade of neuroinflammatory events induced not only following a peripheral nerve injury but in fact to any injury to the PNS/CNS, e.g. traumatic brain- and spinal cord injuries as well as neurodegenerative and neuroinflammatory diseases such as Parkinson’s disease and multiple sclerosis (MS) (Graeber and Streit 2010; Sriram 2011). Some of the alterations seen in reactive microglia includes induction of MHC class I and II antigen (Maehlen et al. 1988; Streit et al. 1989), synthesis of key factors in the complement system (Svensson and Aldskogius 1992; Svensson et al. 1995) as well as synthesis and release of free radicals (Boje and Arora 1992; Li et al. 2005). Microglial cells residing in the CNS are believed to be of mesodermal origin and descendant of the mesenchymal monocyte-macrophage family which during the prenatal and early postnatal period migrates into the CNS where they in the parenchyma transform and differentiate (Ling et al. 1980; Ling and Wong 1993). However, a small number of the reactive microglial cells seen after CNS-injury and peripheral nerve axotomy seem to be infiltrating bone-marrow derived cells (Priller et al. 2001; Ladeby et al. 2005).

Astrocytes

Astrocytes also have a proliferative capacity which is well documented following more severe injuries to the nervous system, however, following a peripheral nerve injury these cells only hypertrophy and fine processes of these cells become located between the perineuronal microglial cells and the neuronal cell membrane (Graeber and Kreutzberg 1986; Tetzlaff et al. 1988; Svensson et al. 1994), reviewed in (Aldskogius 1993; Moran and Graeber 2004).

Oligodendrocytes

Oligodendrocytes seem to be more silent without any significant metabolic or structural changes following peripheral nerve injury, e.g (Watson 1972; Barron et al. 1990; Aldskogius 1993).

3.2 Factors Affecting Degeneration

The severity of the injury and thus the outcome of a peripheral nerve injury depend on several factors. When analyzing data from different studies concerning e.g. neuronal cell death following axotomy it is important not only to identify type and level of injury, but also several other confounding parameters such as nerve model, species, gender and age of experimental animals which may have significant impact on the results.

Avulsion and transection - proximal vs. distal injury

Another crucial factor with impact on neuronal survival is the distance of the injury site to the cell body, related to the amount of trophic support by Schwann cells in the proximal remnant of the nerve (Li and Raisman 1994; Wu et al. 1994; Ide 1996; Cullheim et al. 1999; Chu and Wu 2009; Carlstedt 2011). Avulsion injury (tearing) usually causes proximal breaking of axons near the CNS-PNS interface or even at the CNS side close to the cell bodies and should thus be regarded as a CNS injury (Risling et al. 1983; Carlstedt et al. 1989; Risling et al. 1992). Such injury cause massive cell death as a result of the extreme proximity of the axotomy with total disconnection to the periphery (Koliatsos et al. 1994; Yu 1997; Jiang et al. 2000; Hoang et al. 2003). A transection injury (nerve cut) of a peripheral motor nerve leads to disruption of all of the components of the nerve, nonetheless such an injury is followed by only a low number of motor neuronal loss as long as the two nerve endings are in close contact, although the more proximal the injury is to the site of the localization of the motor neurons, and the greater the distance between the proximal and distal ends of the transected nerve, the more severe consequences will follow (Svensson and Aldskogius 1993; Yu 1997; Itoh et al. 1999; Mattsson et al. 1999). There are thus profound differences in-between the transection and avulsion injuries with regards to regeneration capacity and re-growth of axons.

Nerve model

The type of nerve injured results in different end results, i.e. there is a difference in the vulnerability of the nerve. For example, the hypoglossal nerve seem to withstand axotomies better, as the level of motor neuron loss is less, as compared to the vagal and facial nerve when subjected to identical injuries (Aldskogius et al. 1984; Mattsson et al. 1999; Toyoda et al. 2006).

Species and strain

Not only species but also strain-dependant influences by differences in for example the immune and inflammatory changes after injury exist and have to be considered when evaluating and comparing results (Popovich et al. 1997; Sroga et al. 2003). Thus variations in genetic factors between the same species but different strains can influence outcome. In rats these strain differences are also observed after ventral root avulsion (Swanberg et al. 2006; Piehl et al. 2007).

Gender and age

The age of the animal is strongly influencing the level of motor neuron cell loss and for example it is shown that most of the hypoglossal motor neurons survive in adult animals after a transection injury, while such an axotomy during development and early in postnatal life instead leads to massive depletion of motor neurons (Snider and Thanedar 1989; Fukuyama et al. 2006).

There are also significant differences reported in male and female adult rats in the hypoglossal and facial nuclei, where females were shown to have a twofold greater neuronal cell loss after axotomies (Yu 1988). Furthermore, depletion of sex hormones in male animals elevates cell loss while administration of testosterone to females after injury reduces neuronal loss (Yu 1988; Yu 1989).

3.3 Transmission vs. Growth

An axotomy leads to a genetic switch from a transmitting to a survival mode as genes and proteins related to survival and regeneration are up-regulated (Carlstedt 2011).

Alterations in genes related to e.g. growth factors and cytokines, signal- and receptor pathways, neurotransmitter receptors, transcription factors and cell cycle regulation are found (Yang et al. 2006; Carlstedt 2011). There are differences between peripheral injuries and avulsion of motor nerves where an avulsion leads to up-regulation of genes such as nitric oxide synthase (NOS), p53, Bax, Fas ligand, ANX3/5, TS, ALR and caspase which are associated with apoptosis and DNA damage (Piehl et al. 1998; Martin et al. 2005; Yang et al. 2006; Chu and Wu 2009). These responses also include down-regulation of N-methyl D-aspartate (NMDA) in order to protect the neuron from excitotoxic cell death (Piehl et al. 1995).

Furthermore, there are other factors in the microenvironment stimulating regeneration following avulsion injury and such an axotomy induces changes not only within the neural and glial cellular compartments in the CNS, but also in the extracellular environment in favour for regeneration e.g. cleavage of the growth factor peptide precursors plasminogen activator and matrix-metalloproteinases (Hu et al. 2002). In addition, heat shock protein 27 (hsp27) and several trophic factors and their receptors are up-regulated following the injury (Hammarberg et al. 2000; He et al. 2003; Chu and Wu 2009).

Several of these gene and protein expression alterations are not observed after a peripheral nerve axotomy such as a nerve transection. Here, instead, the machinery sets to only promote survival and regeneration as the motor neurons up-regulate expression of genes that are found to promote neurite outgrowth, axonal regeneration and motor neuron survival such as the low affinity nerve growth factor receptor (p75), alpha calcitonin gene-related peptide (CGRP), the insulin growth factor receptor II (IGFRII), IGF binding protein 6 (IGFBP-6), fibroblast growth factor 10 (FGF 10) and the G-protein-coupled receptor Galanin receptor 2 (Piehl et al. 1998; Yang et al. 2006; Chu and Wu 2009).

The importance of growth- or neurotrophic factors cannot be underestimated in the promotion of survival and regeneration of injured motor neurons. Several families are described to act on motor neurons e.g. the Neurotrophins, the Neuropoietic family, Transforming Growth Factor Beta (TGF- β) superfamily, IGF, FGF, Hepatocyte Growth Factor (HGF), Vascular Endothelial Growth Factor (VEGF), the Serpin superfamily and the Midkine family (Chu and Wu 2009).

The growth factors above have all been found to affect motor neurons during development as well as after injury, however, some of them are found to be of greater importance with regards to the survival and regeneration of the motor neuron after injury. For example, the VEGF family of proteins and their receptors have been shown not only to be an important factor in angiogenesis, but are also found to promote axonal outgrowth (Sondell et al. 2000). After axotomy VEGF is found to be expressed in axotomized motor neurons providing neurotrophic support paralleled by an increase in neuronal survival (Skold et al. 2000; Skold et al. 2004; Pereira Lopes et al. 2011).

Both brain-derived neurotrophic factor (BDNF) from the neurotrophin family and glial cell line-derived neurotrophic factor (GDNF) from the TGF- β family have been found to prevent motor neuron cell loss after ventral root avulsions (Novikov et al. 1995; Novikov et al. 1997; Wu et al. 2003; Yang et al. 2006). BDNF has been found to up-regulate several genes associated with neuronal survival and regeneration, and to down-regulate genes involved in apoptosis that are up-regulated after avulsion.

3.4 Regeneration and Microsurgical Repair

Peripheral nerve injury

In the clinical setting, repair of peripheral nerve injuries include microsurgical techniques such as end-to-end repair with or without tissue adhesives and the use of autografts. Promising experimental techniques include for example synthetic adhesives and Schwann cell containing devices (Osmond 1999; Dvali and Mackinnon 2003; Landegren et al. 2011). In addition, also pharmacological treatment with the calcium flow inhibitor nimodipine has been suggested to promote neuronal survival and regeneration with improved functional outcome (Mattsson et al. 2001; Strauss et al. 2006; Hydman et al. 2009). In addition, the role of physiotherapy in patients suffering from peripheral nerve injury should not be underestimated, as well as the clinical problems related to sensory side of these lesions including pain and dysesthesia. As important as these areas are, further elaboration is beyond the scope of this thesis.

Avulsion injury

Proximal axon disruptions caused by nerve avulsion induce massive motor neuronal cell loss, and requires axonal regeneration across the CNS-PNS border. Thus the

threshold for successful regeneration is far more complex here and involves partial growth through a short distance in the CNS. This involves surpassing myelin associated proteins and the glial scar, which is believed to have importance in sealing off the lesion from the intact tissue in the CNS (Fehlings and Hawryluk 2010). This scar is considered to be formed at least to some part by reactive astrocytes, but also from pericytes (Goritz et al. 2011).

Thus, there is a structural barrier for axonal growth and regeneration after avulsion with axonal lesions in the CNS, but also a biochemical barrier from growth repellant components such as the Nogo protein, chondroitin sulphate proteoglycans (CSPGs), myelin-associated growth protein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) which act inhibitory by interfering with the regulation of axonal growth by inducing growth cone collapse, inhibit neurite growth and contribute to apoptosis (Busch and Silver 2007; Cao et al. 2010).

However, both reimplantation and implantation of a peripheral graft after cervical and lumbar ventral root avulsion promotes the survival of motor neurons and results in axonal regeneration in the CNS across the CNS-PNS transitional zone and into the nerve root or nerve graft, overcoming the non-permissive environment of the CNS. (Carlstedt et al. 1989; Carlstedt et al. 1993; Wu et al. 1994; Cullheim et al. 1999; Hoang et al. 2006; Pinter et al. 2010).

As previously discussed motor neuron survival and regeneration after injury is crucially dependant on support from the periphery, partly provided by proliferating Schwann cells, which by provision of both neurotrophic and neurotropic support contribute to the rescuing of motor neurons and allows for reconnection with the peripheral circuitry. Interestingly, it appears that Schwann cells express different motor and sensory phenotypes which support regeneration that is characterized by the phenotype, in such a way that a motor nerve graft is more favorable for motor neuron survival and regeneration than a sensory nerve graft (Hoke et al. 2006; Chu et al. 2008).

Furthermore, a ventral root avulsion injury induces also intramedullary degeneration of sensory afferent axons and neuropathic pain, on which root reimplantation also seem to have beneficial effects (Bigbee et al. 2007; Bigbee et al. 2008).

The severe avulsion injuries are most often seen in man after brachial plexus injuries and complicated deliveries resulting in devastating functional impairments, and often involves avulsion not only of one but in a majority of cases multiple nerves and roots (Narakas 1985; Havton and Carlstedt 2009). However, experimental findings show that the beneficial effects after a root reimplantation is not confined to the one level of implantation, but effects are also observed on adjacent levels although it appears that the axotomized neuron closest to the implantation site has the highest probability of axonal outgrowth into the implanted root (Hoang and Havton 2006).

4 THE HYPOGLOSSAL NERVE MODEL

The hypoglossal nerve, the XIIth cranial nerve supplies extrinsic and intrinsic muscles of the tongue, except for the palatoglossus muscle. The nerve plays a critical role in eating, swallowing and talking. An injury to the hypoglossal nerve presents with palsy

and atrophy of the ipsilateral muscles in the tongue. Although injuries of this nerve are rare they can include brainstem lesions e.g. after trauma or stroke, more peripheral injuries can be the result after trauma to the neck, hypoglossal nerve schwannomas, head- and neck surgery as well as after internal carotid artery dissections (Mathiesen et al. 2009; Jung et al. 2010; Oyama et al. 2012; Santis et al. 2012).

Although considered a motor nerve, the hypoglossal nucleus is also found to have interneurons and projections from 5- Hydroxyltryptamine (5-HT) neurons in the caudal raphé nuclei, and dysfunction within this system is found to be involved in the sudden infant death syndrome (SIDS) (Kinney et al. 2009).

The hypoglossal nucleus in the brainstem is an upward prolongation of the base of the anterior column of gray substance of the medulla spinalis where sections of the upper part of the nucleus, the hypoglossal trigone, protrudes slightly into the fourth ventricle and the lower part of the nucleus extends downward into the closed part of the medulla oblongata where it lies in immediate proximity to the central canal (Gray 2008). Thus it is close to the ependymal region and the NPCs within this niche.

Furthermore, from an experimental point of view the two hypoglossal nuclei in the brainstem are well defined but still close together on each side of the central canal, making it relatively easy to evaluate morphological changes in the same section from the brainstem having the experimental hypoglossal nucleus in the direct vicinity to the intact control side. The nuclei are also surgically accessible for e.g. microinjections at the floor of the fourth ventricle via a suboccipital, posterior fossa, midline approach, i.e. ideal for being hosts for cell transplantations with minor tissue trauma.

In addition to this, the hypoglossal nerve injury can be considered equivalent to a ventral nerve or root injury.

5 TRANSPLANTATIONS

In the clinical setting transplantation of human fetal dopaminergic mesencephalic fetal tissue to patients with Parkinson's disease was amongst the first where neural progenitors were grafted to the nervous system, and found to have beneficial effects with substantial clinical improvement in some of the patients (Bjorklund et al. 2003). Since the first of these transplantations in 1987 many reports are found on cell-based experimental transplantations to the CNS in injury and disease, not only in rodents, non-human primates but also from several clinical trials on patients (De Feo et al. 2012).

The absolute majority of these have been on spinal cord injuries, degenerative or immune-mediated neurological disorders, or experimental analogues, such as Parkinson's and Huntington's disease, amyotrophic lateral sclerosis (ALS) and MS (Pluchino et al. 2003; Xu et al. 2006; Parr et al. 2007; De Feo et al. 2012; Lu et al. 2012; Riley et al. 2012).

The beneficial effects of transplantations are considered to be of two major principles, first by direct cell replacement of dysfunctional or lost cells but also by a

neuroprotective, or so called bystander effect, e.g. by the production of growth and/or immunomodulating factors (De Feo et al. 2012; Reekmans et al. 2012).

However, grafting of cells to the adult CNS comes with several considerations. A major concern is obviously from where to obtain and harvest the cells to be transplanted and both embryonal, fetal and adult NPCs have been used in allografting and xenografting experiments (Cummings et al. 2005; Parr et al. 2007; De Feo et al. 2012; Lu et al. 2012).

Embryonal or fetal neural progenitors might be preferential due to better proliferation and differentiation abilities when compared to adult cells, but raises some questions and potential limitations. These include all so important ethical and logistical considerations as well as the risk of infiltrative tumor formation the more primitive, or truly stem cell like, the NPC used is (Brederlau et al. 2006).

With allo- and xenografting follows the risk of graft rejection and the concept of regarding the CNS as immune privileged is nowadays questioned, and does definitely not apply to when there is injury or disease affecting the CNS. As discussed previously, microglia are involved in the production of pro-inflammatory cytokines and have the potential to acquire phagocytic and antigen presenting abilities thus, for example, allowing immune cells to recognize epitopes of non-self tissue (Streit et al. 1989; Svensson et al. 1995; Hoftberger et al. 2004; Ekdahl 2012).

NPCs have been found to express MHC class molecules and thus it might be necessary to supply the host with immunosuppressant medication targeting the T cells, e.g. cyclosporine A (Rezzani 2006; Laguna Goya et al. 2011). Hence, giving rise to risks of graft rejection and/or negative effects from additional pharmacological treatment.

To overcome some of the issues stressed above, for example availability and ethical considerations, mesenchymal stem cells have been used in experiments on nervous system repair, and although not found to produce the significant number of neurons as once suggested some beneficial effects have been shown (Parr et al. 2007; Barnabe-Heider and Frisen 2008; Parr et al. 2008).

One promising, and by the Nobel Committee at the Karolinska Institutet appreciated, strategy to be used in grafting for neural repair, is to reprogram fully differentiated somatic cells to become cells that possess pluripotency, i.e. to induce pluripotent cells (Takahashi and Yamanaka 2006; Takahashi et al. 2007).

For apparent reasons a thesis on the topic of stem/progenitor cells and neural repair cannot be presented in December 2012 without elaborating on induced pluripotent stem cells (iPS cells) (Gurdon 1962; Takahashi and Yamanaka 2006).

With this strategy the use of embryonic and fetal tissue is not necessary, as for example a somatic cell such as a fibroblast can be induced to acquire stem or progenitor cell characteristics, giving rise also to neural progenitors to be used for example in experimental research on neural repair and for grafting. Furthermore, immunosuppression is avoided since basically any kind of patient-specific NPC can be generated, which in turn can be differentiated into region-specific neuronal cells as needed (Wu and Hochedlinger 2011; Sommer and Mostoslavsky 2012).

It has also been shown that iPS cells can be generated from adult neural stem/progenitor cells (Kim et al. 2008).

The induction, or reprogramming, in the 2006 Yamanaka experiment included four key pluripotency genes of which one of them was the Sox2 gene, the other three were Oct-3/4, c-Myc and Klf4 (Takahashi and Yamanaka 2006).

With regards to transplantation regimes to the CNS after motor nerve injuries the reports are few, and when it comes to the use of adult NPCs I have not been able to find any papers reporting on this. In two reports a beneficial effect on motor neuron numbers, attributive to a neuroprotective effect by growth factors, have been found (Rodrigues Hell et al. 2009; Su et al. 2009). However in these reports embryonic NPCs and mesenchymal stem cells, respectively, were used as grafts.

In this study we transplant adult NPCs after nerve axotomies in order to evaluate adult cells in a repair strategy, eventually in a model of autografting. By this we hope to reduce many of the concerns with strategies using embryonic, fetal and non-self tissue such as ethical and availability concerns, infiltrative tumor formation risks, graft rejection problems and potential detrimental side effects associated with cell harvesting.

AIMS OF THE STUDY

- I.** Investigate whether hypoglossal nerve transection or avulsion injury that generates low and high neuronal cell death respectively, induces neuro-/gliogenesis from progenitor cells in the ependymal region of the central canal and 4th ventricle.
- II.** Evaluate survival, differentiation and integration of grafted NPCs in a new animal model.
- III.** Determine if survival and integrative capacity of transplanted NPCs was correlated to the extent of the injury.
- IV.** Investigate the potential presence of NPCs in the filum terminale.

MATERIAL AND METHODS

1 ANIMAL EXPERIMENTS

All animal experiments were approved by the local ethical committee on animal research and animal care was in accordance with institutional guidelines. Animals were housed in an environmentally controlled room temperature at $21 \pm 1^\circ\text{C}$ with a 12 h:12 h light/dark cycle, with food and water administered *ad libitum*.

Adult animals were used in all experiments. For experiments in *Paper I* female Sprague-Dawley rats weighing 220-250 g were used. For the experiments in *Papers II and III* Lewis male rats, 300-450 g, were used and in *Paper IV* male Sprague-Dawley rats weighing 400-500 g.

All animals were anesthetized using intraperitoneal injection of medetomidine (Domitor® vet. 1mg/ml, OrionPharma, Espoo, Finland) 0.5mg/kg and ketamine (Ketalar® 50mg/ml, Pfizer Sollentuna, Sweden) 75mg/kg.

1.1 Labeling of Cells in The Ependymal layer (*Paper I*)

The ependymal cell layer was labeled using stereotaxic injection of 20µl 0.1% DiI (1,1'-dioctadecyl-6,6'-di(4-sulfophenyl)-3,3',3'-tetramethylindocarbocyanin) (Molecular Probes/Invitrogen, Eugene, OR, USA) in dimethyl sulfoxide (DMSO) in the lateral ventricle on the right side (0.9 mm posterior and 1.4 mm lateral of bregma, and 3.6 mm below the dura mater) (Paxinos and Watson 1986). DiI is a lipophilic dye and was used in the experiments in order to trace cells. Ten days after DiI administration animals were subjected to hypoglossal nerve (n.XII) injury, see below.

1.2 BrdU Injections (*Paper I*)

In order to assess proliferating cells we used the proliferation marker 5-Bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich Sweden, Stockholm, Sweden). BrdU is a thymidine analogue that is incorporated into the DNA of dividing cells during the S-phase of the cell cycle. The expression can then be detected by the use of an antibody directed against the incorporated analogue. In our experiments 100mg/kg in NaCl

9mg/ml was administered subcutaneously once daily for a maximum of ten days, commencing at the day of injury, see below.

1.3 Hypoglossal Nerve Injury (*Papers I, II and III*)

Two types of axotomies were performed during the experiments that laid the foundation for this thesis; transection injury and avulsion injury of the n.XII on one (the right) side. During transection the whole nerve was cut, leaving two approximate ends, and by avulsion is meant the shear tearing of the nerve from its origin in the brainstem. In both types of axotomies the nerve injury was made at the level where the n.XII passes the external carotid artery on the right side.

1.4 Cell Preparation (*Papers II, III and IV*)

Subventricular zone tissue

For the transplantation experiments subventricular zone (SVZ) neural progenitor cells (NPCs) were obtained from adult Lewis rats (RRRC Missouri University, Columbia, MO, USA (<http://www.nrrrc.missouri.edu>)), carrying an insertion of the enhanced green fluorescent protein (eGFP-transgene) under the Ubiquitin C promoter on chromosome 5. Genotyping was performed by following the genotyping protocol from RRRC-Missouri (<http://www.nrrrc.missouri.edu>). A REDExtract-N-AMP Tissue PCR Kit from sigma (cat# XNAT 100 reactions) was used to extract the DNA from the ear tip biopsy from each animal. Animals that were homozygous for the eGFP transgene were selected as donors, and eGFP non-carrier siblings were selected as the receivers of the transplant (see below).

The rats were 7 to 8 weeks old at day of cell isolation. Cells were isolated in accordance to a protocol described by Johansson and co-workers (Johansson et al. 1999). The cells were propagated for two passages in Dulbecco's modified Eagle's medium/F-12 (DMEM/F12) (Gibco, Life Technologies, NY, USA) containing B27 supplement (Gibco), penicillin (100 U/ml) (Gibco), streptomycin (100µg/ml) (Gibco), epidermal growth factor (EGF) (20ng/ml) (Sigma-Aldrich) and basic fibroblast growth factor (bFGF) (10ng/ml) (R&D Systems Europe Ltd, Abingdon, UK). After the second passage, neurospheres were dissociated and cells were resuspended in a solution that contained 100,000 cells/5µl in DMEM/F12 medium enriched with EGF (20ng/ml) and bFGF (10ng/ml).

For the *in-vitro* differentiation analysis of cell cultures spheres were split after two passages into single cell suspensions, seeded on poly-D-lysine-coated plates, and cultured in medium lacking mitogens but supplemented with 1% fetal calf serum (FCS). The cultures were differentiated for 7 days, and then fixed with 4% paraformaldehyde (PFA).

Filum terminale tissue

Under the operating microscope laminectomies were performed, the conus medullaris identified and filum terminale was dissected in its whole length and extirpated in toto. Thereafter cells were cultured in the same way, and with the same protocol, as described above for the SVZ NPCs. Fixation procedures for cultures were also the same.

1.5 Transplantations (*Papers II and III*)

The non-carrier siblings i.e. wild-type (wt) animals to the eGFP transgenic (Tg) animals, described in section 1.4 above, were the recipients in the transplantation experiments.

Nine days after n.XII injury the animals were reanesthetized and placed in a stereotaxic frame (Kopf®, David Kopf Instruments, Tujunga, CA, USA). During microsurgery the brainstem was exposed and the obex identified. A glass micropipette (Clark Electromedical Instruments, Reading, UK) was pulled to an aperture of 90µm, adapted and sealed onto a Hamilton syringe (Hamilton Company, Bonaduz, Switzerland) and connected to a Univentor 802 syringe pump (Univentor, Zejtun, Malta). At approximately 0.3 mm caudal and 0.1 mm lateral (right) to the obex 100,000 NPCs in 5 µl culture medium DMEM/F12 enriched with mitogens as described above were injected into the right hypoglossal nucleus (nucl.XII). Cells were injected as single cells and the volume was administered at 2 µl/min. The glass micropipette was left in place for 5 min and then withdrawn. Some of the animals received 5 µl DMEM/F12 enriched with mitogens in the same proportions only, i.e. no NPCs were grafted to this group. All the animals received immunosuppressant therapy by injections of cyclosporine 10 mg/kg (Sandimmun® 50mg/ml, Novartis, Täby, Sweden) subcutaneously once daily starting one day prior to transplantation of NPCs or cell medium only injections. After four weeks the cyclosporin was given three times/week.

2 HUMAN MATERIAL

The study on human material (*Paper IV*) was carried out in accordance with the Helsinki declaration, and the experimental procedures were approved by the Stockholm county ethical committee for human research. Tissue from patients was obtained from Karolinska University Hospital only and after informed consent by the patient or parent in accordance with the ethical approval.

Filum terminale (FT) tissue was obtained from young and adult patients (ages 1-60 yrs) (n=21) between the years 2006-2011 (*Table 1*).

Age group	N
1-5	8
6-15	8
>30	5

Table 1. Age of donors, human filum terminale tissue. (From Arvidsson, Fagerlund et al PLoS ONE 6(11), 2011).

All patients underwent MRI scans to exclude tumors and were screened for the presence of infectious disease prior to surgery. The tissue was immediately stored in L15 medium (4°C) in the operating theatre and immediately transported to the laboratory. Connective tissue was peeled off and the FT was mechanically dissociated with scalpels and scissors, and placed in a dissociation medium consisting of 200 U/ml DNase (Sigma-Aldrich) and 0,025% trypsin (Invitrogen) or 10 U/ml papain (Worthington, Lakewood, NJ, USA) in a 37°C water bath for 30 min. Tissue was triturated three times every 5 min, and further incubated. To stop the enzymatic reaction, 10 mg/ml BSA (Sigma-Aldrich) and 10 mg/ml ovomucoid (Worthington) were mixed with L15, added and mixed with the dissociation medium. Cells were collected by centrifugation at 220g for 5 min. To further enrich for progenitor cells, 0.9 M sucrose in Hanks' Balanced Salt Solution (HBSS) (Invitrogen) was added to the tissue solution followed by centrifugation at 750g for 10 min and washing with L15 medium. The cell pellet was resuspended to allow for single cell cultures in 5 or 10 cm diameter petri dishes in neurosphere medium composed of DMEM/F12 (Invitrogen), HEPES (Gibco), B27 supplement (Gibco) and Penicillin-Streptomycin (Invitrogen). The following growth factors were added; 20 ng/ml recombinant human epidermal growth factor (EGF) (R&D Systems) or mouse EGF (BD Biosciences, Franklin Lakes, NJ, USA) and 20 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems). After the first passage, 10 ng/ml recombinant human leukemia inhibitory factor (LIF) (Chemicon/Millipore Sweden AB, Solna, Sweden) was added to the medium. The neurospheres were cultured until they reached a critical size which normally took seven weeks. Another set of cells were cultured in neurosphere medium with the addition of 20% BIT 9500 medium. BIT medium consists of BSA, Insulin (Sigma-Aldrich) and 20 ng/ml transferrin (Stem Cell Technologies, Grenoble, France). Platelet-derived growth factor-BB (PDGF-BB) (R&D systems) was added at 30 ng/ml. Cells were incubated for eight days and the medium was changed three times. Cells cultured with BIT 9500 medium were differentiated on gelatine-coated plates (Sigma-Aldrich). In order to exclude contamination of the data by tumor forming cells, all tissue preparations were screened for the presence of the P53 gene. Cell cultures were fixed with 4% PFA. In order to evaluate the proliferation capacity of FT another set of culture was performed as previously described. After passage the neurospheres were pulsed with BrdU (Sigma-Aldrich) for 48h in the presence of EGF and bFGF. The spheres were placed on coated glasses before fixation in 4% PFA.

3 EXPERIMENTAL GROUPS

3.1 Paper I

A total number of 102 animals were used. 30 animals were subjected to transection injury and 60 to avulsion injury. Survival times were 2, 4, 7, 14 and 28 days after nerve injury (n=6 in the transection group and n=12 in the avulsion group at every time point). 12 animals were sham operated, i.e. skin incision and soft tissue dissection was performed as for the transected and avulsed animals, however no nerve injury was performed. All animals received DiI and BrdU. See *Figure 1* for illustration of experimental setup.

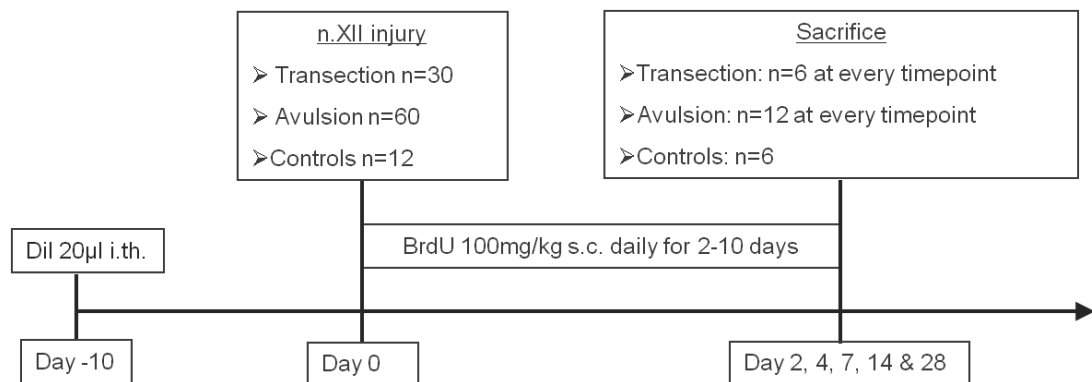


Figure 1. Experimental setup in Paper I.

3.2 Paper II

In this study a total of 104 animals were included of which 8 animals were uninjured controls, which constituted group I. The other three groups were II) injury only, i.e. the n.XII was avulsed, however without injections into the brainstem nucleus, III) injury and injection of cell medium (no cells) to the ipsilateral nucl.XII and IV) injury and transplantation of NPCs to the ipsilateral nucl.XII. Survival times for animals in groups II-IV were 2, 7 and 28 days and 3 months (n=8 in each group and survival time). All animals, also the controls, received immunosuppression. For a schematic illustration of the experimental setup for this paper see *Figure 2*.

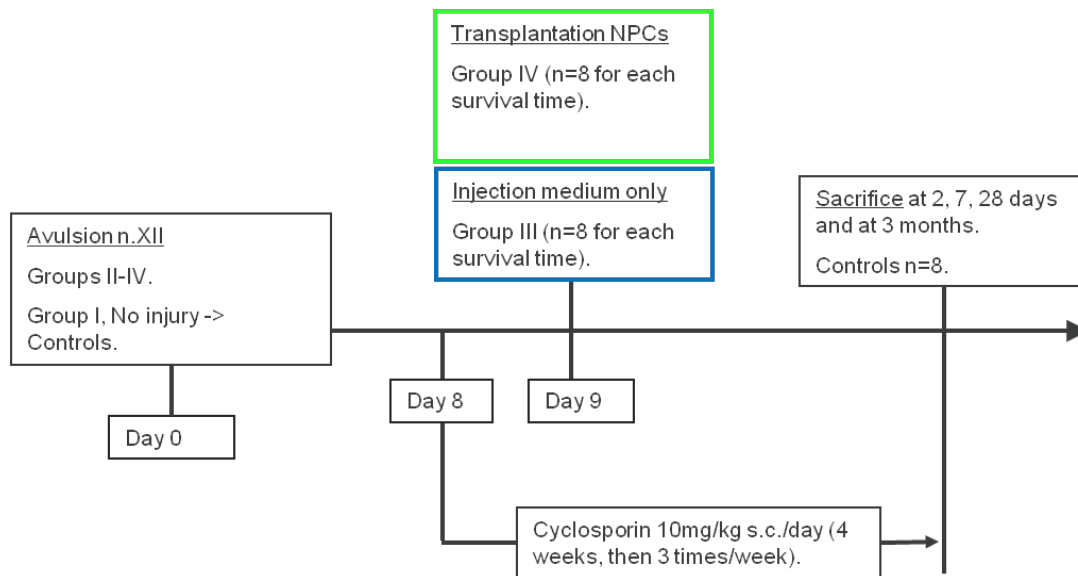


Figure 2. Experimental setup in Paper II.

3.3 Paper III

50 animals were included. The control group (group I) (n=6) was composed of uninjured animals. In the other three groups a n.XII transection injury was performed and the groups were setup in a similar fashion as for *Paper II*, thus the second group included animals with nerve injury but without brainstem injections, group three received cell medium injections into the ipsilateral nucleus of the n.XII after nerve injury and the fourth group received NPCs injected into the ipsilateral nucl.XII after nerve transection. Survival times were 28 days and 3 months, n=6 in each group and survival time. In this study 8 animals with an avulsion injury followed by NPC grafting from *Paper II* were evaluated and included for comparison.

3.4 Paper IV

Human tissue

Patients suffering from tethered cord underwent neurosurgical procedures in order to resolve the tethering of the FT. The total number of patients included in the study was 21, both young and adult (ages 1-60 years). All patients underwent MRI scans prior to neurosurgery in order to exclude tumors and were also screened for the presence of infectious disease.

Animal tissue

A total number of seven animals were included.

4 TISSUE PROCESSING

4.1 Animal Tissue (*Papers I, II, III and IV*)

At sacrifice animals were reanesthetized and transcardial perfusion with body-temperated (37°C) saline followed by cold (4°C) 4% PFA in PBS performed. The brainstems (*Papers I, II and III*), the n.XII on the right side (*Paper III*), tongue (*Paper III*) and the FT (that was not to be prepared for cell culture, see section 1.4 above) (*Paper IV*) were removed and post-fixed for 60-120 min in cold (4°C) 4% PFA in PBS followed by rinse in PBS and cryoprotected for 1 (FT) or 48 hours (brainstems, nerve and tongue) in 15-17 % sucrose (w/v) in PBS. The nucl.XII was serially cryosectioned (14µm) using a Leica CM3000 (Leica Microsystems, Kista, Sweden) cryostat. The tongue was cut into 14µm thick coronal sections and longitudinal cryostat sections (10µm) were made of the n.XII. The FT was cryosectioned in longitudinal and transverse (10µm thick) sections. Tissue was then mounted on SuperFrost® Plus microscope slides (Menzel-Gläser, Braunschweig, Germany).

4.2 Human Tissue (*Paper IV*)

For a detailed description of processing of the human material please refer to section 2 in materials and methods above. Parts of the FT that was not to be prepared for cell culture was fixed in ice-cold 4% paraformaldehyde (PFA) for 24 hours, and cryoprotected in 17% sucrose (w/v) in phosphate buffer (PBS) before being imbedded in Cryomount (HistoLab, Gothenburg, Sweden). The FT was then longitudinally and/or coronally sectioned (10µm thick sections) and mounted as described in section 4.1 above.

5 IMMUNOHISTOCHEMISTRY

Both indirect immunofluorescence and the avidin-biotin complex (ABC) technique were used.

Cell culture sections (*Papers II, III and IV*) were blocked by incubation in PBS with 5% goat serum and 0.1% saponin. All the cryostat sections (*Papers I, II, III and IV*) were first washed in PBS for 30 min before incubation for 60 min in PBS containing 1% bovine serum albumin (BSA), 0.3% Triton-X or 0.1% saponin and 0.1% sodium azide.

All of the primary antibodies used were diluted in these solutions. For staining of dividing cells, S-phase of cell cycle, sections were pretreated with 0.1 M NaOH for 2 min followed by a rinse with PBS (pH 8.5) for 30 seconds and then incubated with antisera to single stranded DNA containing BrdU (Svensson and Aldskogius 1993). Both cell culture and cryostat sections were incubated with one or, for multiple

staining, two or more of the primary antibodies. For the primary antibodies used in this study please see *Table 2*. For negative controls the primary antibody was omitted. All of the sections were incubated with the primary antibody for 24 h at 4°C, rinsed in PBS and subsequently incubated with species-specific secondary antibodies diluted in PBS. See *Table 3* for a list of secondary antibodies.

Some of the sections were also counterstained with the nuclear markers 4',6-Diamidino-2-phenylindole (DAPI) (Invitrogen) (dilution 1:2000) or TO-PRO-3 (Invitrogen) (1:10000). After another step of washing in PBS sections were mounted in Mowiol (Calbiochem, Merck Chemicals Ltd, Nottingham, UK).

Antibody	Species & Type	Dilution	Source
Bassoon	rabbit polyclonal	1:100	Abcam
BDNF	rabbit polyclonal	1:1000	Chemicon/Millipore
BrdU	mouse monoclonal	acc. to supplier	Amersham
ChAT	mouse monoclonal	1:100	Abcam
Doublecortin	goat polyclonal	1:500	Santa Cruz Biotechn.
ED1	mouse monoclonal	1:2000	Abd Serotec
GFP	rabbit polyclonal	1:500	Chemicon/Millipore
GFAP	rabbit polyclonal	1:1000	DAKO
GFAP	mouse monoclonal	1:1000	Chemicon/Millipore
GPx1	goat polyclonal	1:50	R&D Systems
Musashi 1	rabbit polyclonal	1:200	Chemicon/Millipore
Nestin	mouse monoclonal	1:100/1:200	Chemicon/Millipore
NeuN	mouse monoclonal	1:100	Chemicon/Millipore
O4	mouse monoclonal	1:50	Chemicon/Millipore
OX42	mouse monoclonal	1:1600	Harlan SeraLab
p53	rabbit polyclonal	1:400	Santa Cruz Biotechn.
SDF-1 α	rabbit polyclonal	1:50	Santa Cruz Biotechn.
Sox2	rabbit polyclonal	1:200	Chemicon/Millipore
Synaptophysin	rabbit polyclonal	1:100	Abcam
VEGF	rabbit polyclonal	1:50	R&D Systems
Tubulin β -III isoform	mouse monoclonal	1:100	Chemicon/Millipore

Table 2. Primary antibodies used in the study.

For the ABC-technique sections were incubated with biotinylated secondary antibodies (Vector Laboratories, Vector Laboratories Ltd, Peterborough, England) (1:200) for 1 h at room temperature, rinsed in PBS and incubated with ABC (Vectastain® ABC Kit, Vector Laboratories) for 1 h. After another step of rinsing in PBS followed by TRIS-hydrogen chloride buffer (0.1 M, pH 7.45) immunoreactivity was revealed by incubation in 3, 3'-diaminobenzidine (DAB) by using the DAB Substrate Kit for Peroxidase (Vector Laboratories) for 2-10 min. Sections were rinsed in TRIS and dehydrated through a series of graded ethanol's to xylene and mounted in a non-aqueous DPX-medium.

To asses motor neuron profiles in the nucl.XII (*Papers I, II and III*) some of the sections were stained with cresyl violet and dehydrated in a series of ethanol and xylene prior to mounting in a non-aqueous medium (Pertex®, HistoLab) before mounting.

Table 3. Secondary antibodies used in the study.

Secondary Antibody	Dilution	Source
Alexa 488 donkey anti-mouse	1:500	Invitrogen /Molecular Probes
Alexa 488 donkey anti-rabbit	1:500	Invitrogen /Molecular Probes
Alexa 488 donkey anti-goat	1:500	Invitrogen /Molecular Probes
Alexa 594 donkey anti-mouse	1:500	Invitrogen/Molecular Probes
Alexa 633 goat anti-mouse	1:500	Invitrogen/Molecular Probes
FITC goat ant-rabbit	1:200	Jackson ImmunoResearch
Cy3 goat anti-mouse	1:500/1:1000	Jackson ImmunoResearch
Cy3 donkey anti-mouse	1:500/1:1000	Jackson ImmunoResearch
Cy3 goat anti-rabbit	1:500/1:1000	Jackson ImmunoResearch
Cy3 donkey anti-rabbit	1:500/1:1000	Jackson ImmunoResearch
Cy5 goat anti-rabbit	1:200	Jackson ImmunoResearch

6 MICROSCOPY AND QUANTIFICATION

6.1 Confocal Microscopy (*Papers I, II, III and IV*)

Immunolabeling experiments were analyzed by confocal laser-scanning microscopy for visualization of double and triple immunolabeled cells. A Leica TCS SP II system (Leica Microsystems, Heidelberg, Germany) was used and argon-krypton and helium-neon lasers were used to excite the fluorochromes at 488, 543, 594 and 633 nm. Fluorescence was detected with 490-520, 560-630, 610-650 and 640-750 band pass filters, respectively. Images were taken using a 20x (numeric aperture (N/A) 0.7) and 63x (N/A 1.40) objective.

6.2 Widefield Microscopy (*Papers I, II, III and IV*)

Fluorescence was also detected and antibodies assessed by the use of a widefield Leica DM 4000 B microscope (Leica Microsystems) on which also the light microscopy was performed.

6.3 Cell Counting (*Papers I, II and III*)

To carry out a semiquantitative determination of the number of motor neurons (*Papers I, II and III*) 8-10 sections, stained with cresyl violet, from each brainstem of the nucl.XII at the same level for each experimental and control group were analyzed. For this, light microscopy with a 40x objective was used, and neuronal profiles that had a clear nucleus and nucleolus were included at every 168µm throughout the hypoglossal nucleus. The method of Abercrombie was used to correct for volume changes of individual neuronal profiles (Abercrombie 1946; Mattsson et al. 1999). For evaluation of the proliferative response in the ependymal layer (*Paper I*), sections at every 224µm of the nucl.XII were obtained (approximately 8 sections from each brainstem), and cells that co-localized BrdU and DiI in the ependymal layer were included. The numbers of transplanted neural progenitor cells were estimated by counting eGFP-expressing cells in 8 sections from animals receiving grafts, and differentiation was assessed in 2-4 sections for each cell marker (*Papers II and III*). The number of eGFP in the sections was expressed as a percent cell survival of the total number of viable cells injected. This number, i.e. an estimate of viable cells injected, was assessed by the use of Trypan Blue.

7 STATISTICAL ANALYSIS

Data are presented as mean \pm SD. Measurements were followed by statistical analysis by using unpaired two-tailed Student's *t*-test, for multiple group comparison one-way analysis of variance followed by Tukey's post test was implied (*Papers I, II, III and IV*) (GraphPad Prism® 5.0, GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was defined as statistical significance.

SUMMARY OF RESULTS

This is, as stated in the heading, a summary of the results found in the experiments that laid the foundation for this thesis. For a more detailed presentation, please refer to each individual paper.

1 MOTOR NEURON CELL LOSS

The crystal violet staining of motor neuron profiles in the hypoglossal nucleus (nucl.XII) revealed more pronounced cell loss after hypoglossal nerve (n.XII) avulsion injury, than after nerve transection. The differences were observed in both Sprague-Dawley (SD) and wild-type (wt) Lewis rats. In *Paper I* there was a significant decrease in motor neuron profiles in the nucl.XII at 28 days after avulsion when compared to controls. Approximately 43% of the cells were lost, as compared to the transection injury group where we could not observe any significant differences at 28 days when compared to the controls. When examining the wt Lewis animals these differences prevailed (*Papers II and III*) as we found an almost 60% loss of motor neuronal profiles at 28 days after avulsion injury, and as much as an almost 75% loss at 3 months. After transecting the n.XII, only about 10% on average at 28 days and 15% after 3 months were lost.

2 INFLAMMATORY AND GLIA CELL RESPONSE

There are many ways to characterize the levels of inflammation. In this study we assessed this by investigating the levels of microglia (OX42), macrophages (ED1) and the levels of one of the more important antioxidant enzymes, glutathione peroxidase 1 (GPx1).

There was a strong and intense activation of microglial cells after both transection and avulsion of the n.XII which reached a maximum at 7 days after injury (*Paper I*), however no difference in the levels of OX42 could be detected between the two groups. On the other hand we observed differences in the levels of ED1-positive cells (*Paper I*) as well as GPx1-immunoreactivity (*Paper III*). After avulsion injury ED1

was found in the nucl.XII ipsilateral to nerve injury from day 7 post injury and onward, while transected animals displayed very few ED1 labeled cells which were not found before 28 days after the injury. With regards to GPx1 our results showed higher expressions of GPx1 in transplanted NPCs in the avulsion model, when compared to grafting after transection. Also the expression of the GPx1 protein was more intense in the surrounding area adjacent to where the transplanted cells were localized, i.e. in the nucl.XII. This was in contrast to the transection model, where the expression of GPx1 was restricted to the cells.

This would indicate that in addition to the differences in motor neuron profiles after the two types of injury there is also a difference in the levels of inflammation, although we were not able to detect any significant differences in microglial levels. The astrocytic response (assessed by GFAP expression) was profound after both transection and avulsion and reached the highest levels at 14 days after injury after which a decline was seen (*Paper I*).

3 ENDOGENOUS PROGENITOR CELL ACTIVATION

All of the results in this section were obtained from Paper I.

In the immediate vicinity to the nucl.XII Sox2-positive cells were found in the ependymal region in the central canal and fourth ventricle in the brainstem. The expression was abundant. Quantification of Sox2 expressing NPCs did not reveal any differences between transection and avulsion injury. However, Sox2-positive cells were also found subependymal to the ependymal layer and there was a dorsal-ventral polarization of the Sox2 cells with significant more cells in the middle and dorsal parts of the central canal region, as compared to the ventral part.

When we investigated the proliferation of these cells after avulsion injury BrdU-immunoreactivity was confined almost exclusively to the dorsal parts and increased from 4 days, progressed at 7 days post injury after which a decline was observed at later survival times. The increase was significant when compared to control animals. This increase in proliferation was not seen after nerve transection.

After avulsion, but not transection, Nestin was expressed in the ependymal layer at 7 days after injury. In avulsed animals DiI-labeled cells were detected in the nucl.XII ipsilateral to nerve injury, indicating migration from the ependymal layer. The migrating DiI-positive cells expressed GFAP and some of them also showed immunoreactivity for BrdU. We could not detect cells expressing neuron or oligodendroglia markers. No migrating cells were observed in the contralateral, uninjured, nucl.XII.

In the avulsed animals we were able to find the chemotactic cytokine SDF-1 α in the, to the injury, ipsilateral nucl.XII. SDF-1 α was expressed in GFAP-positive cells and observed from 4 days after injury with a gradual increase at later survival times. Again, this was not found in controls or in transected animals.

Thus our findings indicated proliferation, migration and differentiation of ependymal region NPCs after avulsion injury, but not transection, showing that there is quite a difference in endogenous NPC activation between the two models of axotomy. However, in these experiments neuronal differentiation failed.

4 TRANSPLANTATIONS

All of the results in this section were obtained from Papers II and III.

4.1 Characterization of Subventricular Zone Progenitor Cells

In order to assess stemness and differentiation potential of the cells aimed for transplantation, we categorized the cultured cells that were harvested from the SVZ of the eGFP transgenic (Tg) Lewis animals. Before differentiation approximately 90% of the cells expressed Sox2 indicating they were NPCs. After differentiation for 7 days *in-vitro* the cells possessed the ability to differentiate into neurons (2-4%), oligodendrocytes (35-40%) and astrocytes (57-61%).

We concluded that cells from the SVZ of adult Lewis eGFP Tg animals had the same abilities as has been established for cells from the SVZ in wild-type animals.

4.2 Viability of Cells Prior to Transplantation

In order to assess the viability of cells aimed for grafting we used trypan blue, which traverses the cell membrane in a dead cell but is not absorbed in a viable cell. This showed that 85-90% of the cells were viable before transplantation.

4.3 Survival, Differentiation and Integration of Cells

Transplanted cells were detected by their eGFP expression. The identity of the cells as grafted NPCs was also confirmed with the use of the non-fluorescent ABC-technique where an antibody directed against GFP was applied. The majority of cells were found in the nucl.XII ipsilateral to nerve injury, although cells were also seen in the trajectory channel.

At the early time points, 2 and 7 days (*Paper II*), grafted cells appeared small and round, without morphological differentiation and were not expressing phenotypic markers for neurons, oligodendrocytes or astrocytes. When assessed at 3 months we estimated that as many as 10% of transplanted cells were present, however the number was highly variable between subjects. Numerous of the grafted cells in the nucleus of the avulsed n.XII displayed morphology characteristics for neurons, including long extensions indicating axons and dendrites. Some of these cells were found to express Tuj-1, we estimated this number to 5.1% of the total number of transplanted eGFP NPCs, and some of them were found to also express NeuN.

However, the majority of grafted cells, more than 60%, were co-labeled with GFAP indicating differentiation towards the astrocytic lineage. Although present, few of the transplanted cells (<1%) expressed oligodendrocyte markers, and these cells tended to maintain a round, immature, appearance although some of them displayed a spreading, plane morphology. These findings were in contrast to the results we obtained from transected animals. In these animals transplanted eGFP cells appeared small and round with an immature morphology also at 3 months. The cells did not display immunoreactivity for neurons or oligodendrocytes and the number of GFAP-positive eGFP cells was much less, only about 15%. The total estimated number of eGFP cells at 3 months in the hypoglossal nucleus as compared to the total number of transplanted cells also differed between these two injury groups; in the avulsion group 10% of cells were detectable as compared to less than 2% in the transection injury group. Another difference between the groups was that 25-30% of the eGFP cells still expressed Sox2 in transected animals, indicating that these cells were still in a neural progenitor cell state. Sox2 was not found in eGFP cells in the avulsed animals. In order to determine integration of grafts in the nucl.XII we applied a protocol for labeling with markers for synaptic connectivity. In this study synaptophysin and bassoon. These two markers were found to be present around the transplanted cells and along the dendrite-like extensions (synaptophysin) and also around the grafted cell bodies (bassoon) in the nucl.XII after avulsion injury but not transection. Thus we interpreted this to indicate that eGFP NPCs were integrated after transplantation to animals with nerve avulsion injury, but not transection.

4.4 Effects on Motor Neuron Survival

As many motor neurons were lost in the nucl.XII ipsilateral to nerve avulsion we assessed whether the transplanted NPCs could increase the number of motor neurons, either by differentiation into motor neurons, thus replacing the ones lost, or by reducing the loss of motor neurons. This was also compared between the two axotomy models.

In none of the papers were we able to detect differentiation into motor neurons as we were not able to find ChAT-immunoreactivity in eGFP NPCs, neither could we detect cells with motor neuron morphology that were expressing eGFP. However, there was a significant increase in number of motor neuron profiles in the nucl.XII after nerve avulsion in animals receiving NPCs. After NPC transplantation in these animals 55% of motor neurons were present at 28 days and 46% at 3 months and displayed morphology as of the motor neuron profiles in controls. The numbers were significant when compared to avulsed animals without grafting where only 43% and 24% of motor neuron profiles were present at 28 days and 3 months respectively and, notably, also to animals receiving injections of cell medium only to the nucl.XII. Transplanted NPCs thus were able to reduce motor neuron cell loss after avulsion injury, and were found to express the neurotrophic factor vascular endothelial growth factor (VEGF) after grafting to the nucl.XII, as well as *in-vitro*. In addition, we found intense expression of the antioxidant GPx1 (see also section 2 above) in the eGFP

NPCs in the avulsed group. We suggest that this up-regulation of GPx1 is crucial for their, and also the motor neurons, own survival in a microenvironment with local accumulation of inflammatory compounds and free radicals.

GPx1 expression was also found after grafting of NPCs to transected animals, however not as intense.

When compared to the results obtained after avulsion injury of the n.XII, the number of motor neurons in the nucl.XII was not influenced by the graft after transection of the nerve.

As a transection injury would provide better conditions for axon regeneration we investigated whether the transplanted cells had the ability to form regenerating axonal projections. We extirpated and examined the n.XII from its exit point at the brainstem to its projection into the tongue but were not able to detect green fluorescent axonal structures within the nerve, neither were we able to detect signs of eGFP in the tongue, indicating that signals for axonal growth from our graft were lacking, or at least insufficient.

5 PROGENITOR CELLS IN THE FILUM TERMINALE

All of the results in this section were obtained from Paper IV.

5.1 Distribution

Sox2 and Mushashi-1 were applied on sagittal and/or coronal sections of the human filum terminale (FT). Sox2-immunoreactivity (IR) was abundant in the FT and labeled cells were found in the ependymal cell layer surrounding the central canal. Mushashi-1-IR was also detected in ependymal cells surrounding the central canal as well as in the subependymal compartment where cells were found in organized layers but also scattered in larger clusters at the surface of the FT. Cells positive for Mushashi-1 were also localized in smaller islets at a distance from the central canal. Mushashi-1 expressing cells were found to express Sox2 and cells expressing strong immunolabeling for Sox2 were found to express GFAP.

In longitudinal sections from the rat FT we confirmed the presence of an ependymal-cell-like lined central canal where the cells expressed Nestin.

In the human the central canal in the FT displayed more of a scattered system of tubular structures lined by the ependyma, in contrast to the rat FT where the central canal was large and appeared better defined.

5.2 *In-vitro* Characterization

Single cells from the human FT tissue formed neurospheres in 13 out of 21 patients (62%). We observed that neurosphere formation was more frequent from young patient, showing excellent growth capacity allowing for up to 15-30 passages to be possible. Although we were also able to propagate cells from older patients the FT

tissue from here showed a lower growth rate when compared to the young (*Figure 3, Table 4*). Single cells isolated from the neurospheres were able to give rise to secondary neurospheres, thus demonstrating that at least some of the cells possessed self-renewal capacity. In cells at early stages Nestin immunolabeling was present. When allowed to differentiate we found that 5% of the cells expressed β -III-tubulin, this expression was validated in some of the patients for each expansion of the cells and found to be maintained for up to 29 expansions. When platelet-derived growth factor-BB (PDGF-BB) was added to the differentiation medium we observed a strong, almost tenfold, increase in the numbers of β -III-tubulin expressing cells. Upon differentiation also GFAP-positive cells with a morphology characteristic for astrocytes appeared.

Rat FT cells were also cultured and we were able to obtain neurospheres from 5 out of 13 cultures (39%). However, due to the small amounts of harvested tissue, cells from rat FT was exceptionally difficult to expand, and thus to further analyze.

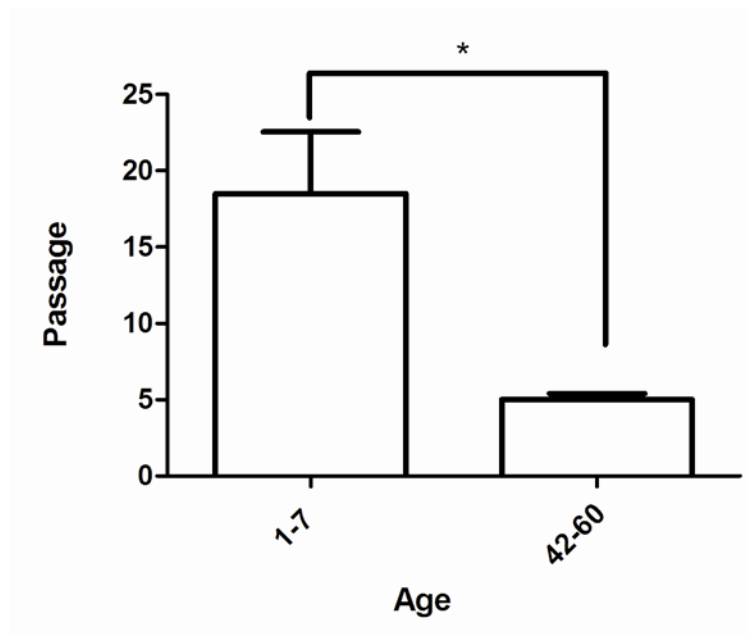


Figure 3. Quantitative determination of cell expansion from young donors (1-7 years) and old donors (42-60 years). (From Arvidsson, Fagerlund et al PLoS ONE 6(11), 2011).

Age group	n	spheres	no spheres
1-5	8	6	2
6-15	8	4	4
>30	5	3	2

Table 4. Age of donors and the number of neurospheres formed. (From Arvidsson, Fagerlund et al PLoS ONE 6(11), 2011).

DISCUSSION

Before summarizing the conclusions in Roman numerals the following section will discuss the findings and why certain conclusions can be made. The discussion will be both specific and general. I will also within this part include some methodological concerns that should not be neglected, and elaborate on future perspectives. The discussion aims at integrating all of these components.

1 INTRODUCTION TO DISCUSSION

In the first three papers (*Papers I, II and III*) we studied the fate of adult endogenous and grafted neural progenitor cells (NPCs) in the CNS after peripheral nerve injury. The overall aim was to challenge the hypothesis that endogenous or grafted NPCs will respond to degenerative events in their microenvironment directing their fate towards replacing lost cells.

The scientific approach used to test the hypothesis is based on two different models of axotomy (transection vs. avulsion) of the hypoglossal nerve (n.XII) that generates fundamental differences concerning cell death of affected motor neurons. Transecting (cutting) the hypoglossal nerve using a pair of fine scissors in the periphery cause only modest neuronal degeneration in contrast to avulsion injury that induces massive cell death in the ipsilateral hypoglossal nucleus (nucl.XII) in the brainstem.

We found a significant activation of endogenous as well as grafted NPCs in terms of proliferation, migration and differentiation following n.XII avulsion injury. However, the cells remained silent following a transection injury. These findings support the hypothesis that degenerating neurons are part, of what is likely a series of events, which eventually results in formation of signaling cues triggering the response of the NPCs. Alternative explanations could be related to co-variables like neuroinflammatory events that are more pronounced following avulsion injury.

The activation of the NPCs most likely depends on both levels of cell degeneration, death and neuroinflammation which are closely interrelated and together contribute to form a highly complex microenvironment in the affected region.

The rationale for using the hypoglossal nerve system for these experiments is based on several issues. First, the n.XII is well characterized with regards to events occurring

in the peripheral nerve as well as in the corresponding nucleus in the brainstem after axotomy. These biological events varies depending on the type of injury etc. (see introduction), but includes always, at least to some degree degeneration and death of motor neurons, glial responses and neuroinflammatory events.

Thus, the hypoglossal system as an experimental arena is well characterized and established and frequently used to investigate degeneration and regeneration in the nervous system *in-vivo* (Aldskogius et al. 1980; Aldskogius et al. 1984; Snider and Thanedar 1989; Svensson 1993; Svensson et al. 1995; Yu 1997; Itoh et al. 1999; Jiang et al. 2000; Toyoda et al. 2006).

Furthermore, the nucl.XII is positioned in immediate vicinity to the central canal and fourth ventricle in the brainstem, with short distance to the ependymal region with the residing NPCs. Therefore, this experimental setup allows for studying the role of degenerating neurons on adult NPCs in the CNS following a peripheral nerve injury *in-vivo*. An area in which little is known.

The precise nature of the immature cells in the ependymal region of the central canal and fourth ventricle that are referred to as NPCs in this study is still controversial and debated in the literature. Although it appears that ependymal cells, which during normal conditions are quiescent, are not the true stem/progenitor cells from which all adult neurogenesis has its origin, cells in the ependymal region throughout the adult CNS definitely have progenitor cell characteristics in the sense that they can be activated upon injury and disease and give rise to neurons, oligodendrocytes and astrocytes (Doetsch et al. 1999; Johansson et al. 1999; Namiki and Tator 1999; Brundin et al. 2003; Mothe and Tator 2005; Danilov et al. 2006; Coskun et al. 2008; Meletis et al. 2008; Carlen et al. 2009; Cizkova et al. 2009; Foret et al. 2010).

To identify the NPCs we used markers that are expressed by stem/progenitor cells during development; Sox2 and Nestin (Hockfield and McKay 1985; Dahlstrand et al. 1995; Bylund et al. 2003; Graham et al. 2003). In the adult there is no single exclusive marker for the stem/progenitor cells, but these two are considered to be expressed also in the adult CNS by NPCs, as defined in this study, and together with Mushashi-1 were the markers we used for identifying the neural progenitors (Doetsch et al. 1997; Kaneko et al. 2000; Filippov et al. 2003; Ellis et al. 2004; Komitova and Eriksson 2004; Baer et al. 2007).

Although we did not fully characterize the cells, as we for example did not use other markers or electron microscopy, we considered the cells to be NPCs.

Furthermore we cannot specify the exact identity and true nature of the subventricular zone (SVZ) NPCs that we harvested from the lateral ventricle wall; these could very well be the type B SVZ astrocyte-like cell, ependymal cells or also to some extent the type C cell. And, of course also a combination of these cell types. However, again the cells possessed NPC features as defined for the investigations in this study.

Hypoglossal nerve injury is not often seen in clinical practise although it occurs occasionally following tumour surgery, trauma etc (Jung et al. 2010; Oyama et al. 2012; Santis et al. 2012). However, the main aim of this part of the study was to

investigate, assess and evaluate the neurobiological events with regards to NPC activation after a motor nerve injury. Importantly though, with reference to clinical implications, the model can be considered to be comparable to a ventral nerve and root axotomy, thus making the neurobiological phenomena we observed clinically highly relevant and applicable to the often devastating ventral root avulsion injuries (Havton and Carlstedt 2009).

2 ENDOGENOUS PROGENITOR CELLS

2.1 Distribution

We found abundant expression of Sox2 in the ependymal region in the central canal and fourth ventricle along the rostro-caudal extent of the nucl.XII.

Sox2 has been elaborated on earlier; however since it is a key marker in this study some further discussion is warranted.

The Sox proteins (Sex Determining Region of Y chromosome (SRY)) are HMG-box related, that is they belong to the High Mobility Group family of proteins that act as transcription factors which all have similarities within their HMG domain DNA-binding sequence (Lefebvre et al. 2007; Chew and Gallo 2009). More than twenty different Sox proteins have been found and based on the similarities within the HMG domain they are divided in nine groups (A-H) (Lefebvre et al. 2007; Chew and Gallo 2009). The B1 group contains Sox1, Sox2 and Sox3 which are shown to have importance in specification and maintenance of cells in a neural stem or progenitor cell state during development, but importantly also in the adult CNS (Bylund et al. 2003; Graham et al. 2003; Ellis et al. 2004; Komitova and Eriksson 2004; Baer et al. 2007). Other Sox proteins play important roles in further specification, such as the Sox4, Sox11 and Sox12 proteins, which have been demonstrated to be important for the establishment of neuronal properties, and the Sox 8, 9 and 10 proteins which are involved in regulation and formation of oligodendrocytes and Schwann cells i.e. myelinating cells (Bergsland et al. 2006; Dy et al. 2008; Chew and Gallo 2009).

We chose Sox2 as a marker since it is shown to be expressed in neural stem or progenitor cells from very early on in development and prevails in the adult CNS as it is continuously expressed in the adult NPCs, which also the Sox1 and Sox3 proteins are (Ellis et al. 2004; Komitova and Eriksson 2004; Baer et al. 2007; Wegner 2011). However, Sox2 is one of the more extensively studied.

By no means is one single transcription factor in control of cell fate decisions and differentiation, instead several different transcription factors from various families interact. Importantly though, the Sox proteins have a key role in keeping cells in an NPC state, controlling differentiation and cell fate and appear to be of paramount importance in coordinating the sequential gene expression in neurogenesis (Bergsland et al. 2011; Wegner 2011). Notably, it is also one of the transcription factors, together with a set of others, that has the ability to re-establish pluripotent cell characteristics in cells that are terminally differentiated (Takahashi and Yamanaka 2006).

We demonstrate that Sox2 expression was not only confined to cells in the ependymal layer, but we also detected several cells located in deeper layers that were expressing Sox2. These findings are supported by others, and it appears that not only cells in the ependymal layer of the central canal are Sox2 positive, but also cells in a SVZ resembling subependymal layer (Hamilton et al. 2009; Foret et al. 2010).

This region of the CNS does not contain a SVZ of the same dignity as in the forebrain, but there is a layer of cells subependymal to the central canal lining which interestingly has also been found in humans (Doetsch et al. 1997; Dromard et al. 2008). Thus a SVZ-like niche is present also within here (Hamilton et al. 2009).

Quantification of Sox2 expressing cells revealed a difference in NPC numbers between the ventral, middle and dorsal parts of the central canal. Significant more Sox2-positive cells were found in the dorsal and middle regions when compared to the ventral part. Furthermore, avulsion injury induced proliferation in the ependymal layer which was also confined to the more dorsal parts of the central canal, i.e. not ventral. This dorsal to ventral gradient has been found also by others where the majority of proliferation markers were expressed dorsally, both in the ependymal as well as in the subependymal layer, although the majority of dividing cells were located in the ependymal layer (Hamilton et al. 2009). However, some report on an equal distribution of dividing cells for example after spinal cord injury, and others that the majority of proliferative cells are instead found in the medial part of the central canal, i.e. not dorsal, after physiological stimulation (Mothe and Tator 2005; Cizkova et al. 2009).

The difference in dorsal-ventral activity of the ependymal layer in the central canal could be correlated to the dorsal-ventral patterning during developmental neurogenesis, which is important for the production of different neuronal cells (Cizkova et al. 2009). And importantly, as has been discussed previously within this thesis, regulation of adult neurogenesis shares several signaling pathways with neurogenesis in development (see introduction). During development the morphogen Sonic hedgehog (Shh) is secreted from the notochord and induces the floor plate of the neural tube (Ulloa and Briscoe 2007). This initiates ventralization and a concentration gradient of Shh is produced that induces the formation of different progeny by a concentration and time dependant mechanism. The cells of the floor plate eventually also express Shh, and the highest levels of Shh are found in the ventral part. Within here five separate neuronal progenitor domains are formed, which gives rise to motor neurons and interneurons, but also has a critical role in the development of oligodendrocyte precursors (Dessaud et al. 2008).

Furthermore, in dorsal-ventral patterning, the Bone morphogenic proteins (BMPs) secreted from the ectoderm leads to the formation of the roof plate. BMP is expressed also in the roof plate and the BMPs are found in the dorsal part, hence the concentration is highest here. This signaling has an important role in dorsal-ventral axis patterning, and together with Shh the dual morphogen gradient specifies different subclasses of neurons depending on their dorsal-ventral position and the concentration of Shh and BMP (Ulloa and Briscoe 2007). BMP thus promotes dorsal

patterning and formation of dorsal phenotypes such as sensory interneurons. However, BMP signaling is exceptionally complex, with a large number of functions in neural stem/progenitor cell fate and differentiation at several stages of neural development, therefore the effects differs with and are also tightly regulated in space and time (Bond et al. 2012).

Shh and BMP signaling are active and have important functions also in adult neurogenesis. Shh promotes proliferation and maintenance of the neural precursor cell (Ahn and Joyner 2005; Favaro et al. 2009). BMPs and their signaling systems are involved in and act as regulators of self-renewal, proliferation and differentiation of NPC contributing to the maintenance of a neurogenic niche in the adult (Lim et al. 2000; Quadrato and Di Giovanni 2012).

It is difficult to specify exactly what this dorsal-ventral differences we, and others, have observed means in the adult. It appears however, that in this region activity upon injury does not involve extensive proliferation of the part that during development give rise to motor neurons i.e. the ventral region (Ulloa and Briscoe 2007).

2.2 Proliferation

Assessing proliferation has been of fundamental importance for the acceptance of adult neurogenesis, and although not fully recognised at that time, it was by the use of radioactively labeled Thymidine which integrates in the DNA during mitosis Joseph Altman showed that new neurons were formed also in the adult CNS (Altman 1963; Altman and Das 1965). Since then adult neurogenesis has gained general acceptance and strongly contributing to this are technical developments. In this, the application of the BrdU method, as we used, in proliferation assays is one strongly contributing factor.

BrdU, or 5-Bromo-2'-deoxyuridine, is a Thymidine analogue which is incorporated in the DNA of dividing cells during the S-phase of the cell cycle, and is a robust marker for proliferation (Kee et al. 2002). As it is an exogenous agent it has to be administered to the experimental object by which follows uncertainties regarding levels of concentration, and also potential side effects such as cytotoxicity (Kee et al. 2002). Because of this, it is possible that the numbers of proliferating cells are not adequate, although if erratic they are most likely underestimated. Not to be forgotten though is that it has been found to also label cells undergoing DNA repair, i.e. not dividing cells and this, the above and also other aspects has to be kept in mind when using BrdU (Kuhn and Cooper-Kuhn 2007). However, there is no doubt that BrdU is indeed useful and since the incorporation into the DNA is brought on to the daughter cells, it can also be used to trace cells.

In accordance with the quiescent nature of the ependymal cells we only occasionally detected BrdU-incorporation in our controls (Coskun et al. 2008; Meletis et al. 2008; Carlen et al. 2009; Barnabe-Heider et al. 2010).

The transection injury did not induce an increase in proliferative activity in the ependymal layer, indicating lack of or insufficient signals.

This was in contrast to the avulsion injury which induced a significant increase in proliferation. We show that there is a temporal profile in this proliferation which reflects both the need for self-renewal, thus maintaining a pool of NPCs in the niche, as well as producing glial progeny that for example contribute to the formation of the glial scar after CNS injury, contributing to tissue integrity (Namiki and Tator 1999; Mothe and Tator 2005; Meletis et al. 2008; Cizkova et al. 2009; Barnabe-Heider et al. 2010).

It is well documented that the ependymal cell can respond to injury with quite a dramatic increase in proliferative activity, should the stimulus be strong enough and that a more prominent stimulus induces a more excessive ependymal cell response in the central canal region (Meletis et al. 2008; Cizkova et al. 2009; Barnabe-Heider et al. 2010; Foret et al. 2010). However, it is also suggested that an even stronger stimulus, e.g. more severe injury, does not necessarily elicit a more intense response (Namiki and Tator 1999). As long as the injury or disease is severe enough to reach the threshold for signals to induce activation, the cells respond.

Both of these two findings can be considered to support our findings on transection vs. avulsion and the influence on ependymal layer cells (Namiki and Tator 1999; Cizkova et al. 2009; Foret et al. 2010). To be mentioned is that also non-glial cells contribute to the scar formation and it is becoming clearer that pericyte derived cells have an important role here (Goritz et al. 2011).

2.3 Migration and Differentiation

In order to fully study NPC activation in the brainstem ependyma including migration and differentiation from the ependymal region, we obviously needed to be able to follow the cells as migration and differentiation proceeded, by other means than by BrdU-incorporation and intrinsic marker expression as the dynamic in gene transcription would change the phenotypic characteristics of the cell.

Due to the direct contact of the brainstem ependyma NPCs with the cerebrospinal fluid (CSF) we were able to mark the progenitors by an intrathecal injection of the lipophilic dye DiI.

One could argue that the use of DiI for the use of migratory and differentiation assessments is to some extent uncertain as, for example, cell to cell transfer of the dye from the ependymal layer cells would result in staining of other cells thus leading to misinterpretations of the findings. This is of course possible, however previous researchers have elaborated on this issue and found such a phenomenon not so likely, and there are several papers on the use of DiI as a method for labeling of cells for the same purpose as ours (Johansson et al. 1999; Brundin et al. 2003; Mothe and Tator 2005; Danilov et al. 2006).

Obviously, other methods are available and maybe superior, such as retroviral labeling, or the use of transgenic animals with reporter expression of certain specific markers (Johansson et al. 1999; Meletis et al. 2008). However, also these techniques come with considerations when interpreting results, as recombination is seldom complete thus making recombination efficacy important.

In addition to proliferation, the avulsion injury induced migration of DiI-labeled ependymal layer cells to the ipsilateral nucl.XII.

Chemotactic signaling systems have been shown to be active also in the brainstem nuclei, including the nucl.XII, after a motor nerve axotomy (Harrison et al. 1998; Ji et al. 2004). Microglia express the chemotactic cytokine (chemokine) receptor CXCR4 which is the receptor for the chemokine SDF-1 α (Tanabe et al. 1997). Interestingly CXCR4 has been found on NPCs in the adult CNS and CXCR4/ SDF-1 α signaling is involved in the migration of NPCs (Tran et al. 2004; Thored et al. 2005; Kokovay et al. 2010).

This correlates to our findings where we show a time dependant migration of NPCs from the ependymal layer concomitant with an increase in expression of SDF-1 α in the nucl.XII ipsilateral to nerve avulsion.

In our study we encountered the SDF-1 α in GFAP expressing cells of astrocytic morphology, which has been found also by others (Xu et al. 2007; Moll et al. 2009). There are however reports stating that cells in the ependyma are negative for CXCR4 and that SDF-1 α /CXCR4 signaling is not likely to mediate attraction of ependymal layer, or their progeny, cells at least in a spinal cord lesion (Meletis et al. 2008).

Upon migration to the nucl.XII the endogenous NPCs differentiated into cells of astrocytic lineage but we were not able to detect neuronal differentiation.

This gliogenic fate of the ependymal layer cells is well documented and it seems that the microenvironment in this region favors glial instead of neuronal differentiation in response to injury (Johansson et al. 1999; Shihabuddin et al. 2000; Brundin et al. 2003; Mothe and Tator 2005; Meletis et al. 2008; Cizkova et al. 2009). However, although endogenous neurogenesis mostly seem to fail in this region the ependymal layer NPCs of the central canal are found to have the capacity to form neurons upon stimuli (Danilov et al. 2006). Interestingly, in this report on an experimental model of multiple sclerosis (experimental autoimmune encephalomyelitis (EAE)) neurogenesis appears to have been generated from the dorsal parts, in proximity to where the area of neuroinflammation was found i.e. the dorsal column. And, although speculative, as we also report on a dorsal-ventral polarization in proliferation, it could be so that signals for ventral central canal ependymal layer cell activation in response to avulsion injury are lacking or insufficient. Therefore no NPCs patterned for motor neuron differentiation were activated, and hence we did not find markers for newly formed neurons.

To summarize this part, although we failed to show neurogenesis, we demonstrate that this cell pool is activated after avulsion injury including proliferation, migration and glial differentiation after avulsion injury. Another component indicating activation in the study was our, and others, finding that Nestin is re-expressed in the ependymal layer after injury. This probably reflects the stem/progenitor cell feature of these cells (Dahlstrand et al. 1995; Frisen et al. 1995; Namiki and Tator 1999; Mothe and Tator 2005; Foret et al. 2010). Again, this was seen only after avulsion of the n.XII. Therefore our findings clearly show that activation of the endogenous NPCs in

the ependymal layer of the central canal and fourth ventricle occurred after avulsion injury only, not transection.

3 GRAFTED ADULT PROGENITOR CELLS

Transplanted adult SVZ NPCs were found to respond more promptly to a severe n.XII injury. Thus, the results on grafting were consistent with the findings on endogenous NPCs as both were activated in a more extensive way after avulsion. Importantly though, the grafted cells differentiated also to neurons and appeared to have integrated with the host neurons. Furthermore, the SVZ NPCs exerted a striking beneficial effect on motor neuron numbers in the nucl.XII after avulsion injury. Significant more motor neurons were present at both 28 days and 3 months after injury post transplantation when compared to controls. This effect was not found after grafting to transected animals.

In order to evaluate whether this truly was attributable to the transplanted cells we performed experiments where cell medium only, i.e. no NPCs, was injected into the nucl.XII after axotomies.

Cell medium injections had no effect on motor neuron survival. We therefore concluded that the increase in motor neuronal numbers was attributable to the transplanted SVC NPCs.

There could be two principally different explanations for this, either by the formation of new motor neurons from the grafted cells, i.e. direct cell replacement. Or, by a neuroprotective effect, a so called bystander effect (De Feo et al. 2012). In the former we would have found evidence for differentiation of NPCs to motor neurons in the nucl.XII, and although we found neuronal differentiation we could not detect motor neuronal markers in eGFP NPCs. Neither could we detect eGFP in cells with motor neuron profiles. Therefore, we concluded that the significant increase in motor neurons had to be due to a neuroprotective effect by the production of growth and/or immunomodulating factors (De Feo et al. 2012).

We found that grafted NPCs expressed both the antioxidant enzyme glutathione peroxidase 1 (GPx1) and the growth factor vascular endothelial growth factor (VEGF).

The intense GPx1 expression in transplanted adult SVZ NPCs seem to reflect these cells protective alertness, and is probably crucial for their survival in a microenvironment with ongoing and extensive cell degeneration that follows a severe injury with local accumulation of free radicals and oxidative stress. And, in part explains the effect on motor neuron survival that we demonstrate.

In traumatic injuries and inflammatory disease of the nervous system there is activation and proliferation of microglia, also after a motor nerve injury, and abundant production of nitric oxide (NO) (Novikov et al. 1995; Novikov et al. 1997; Yu 1997; Aldskogius 2001; Moran and Graeber 2004). Although we did not directly investigate levels of NO we found an intense expression of GPx1 not only in grafted cells, but also widely distributed in the nucl.XII after avulsion. After transection the

expression was low, in accordance to the low levels of motor neuron degeneration and death in this injury.

The GPx1 expression correlates to levels of oxidative stress as transcription and expression of the GPx1 gene and enzyme presumably is higher with increasing levels of inflammation, microglia activation and NO production i.e. in an on behalf of the motor neuron hostile environment.

The correlation is not absolute, obviously, but the free radical NO and the effects it has is largely mediated through reactive oxygen species (ROS), reactive NO species (RNOS) and free radicals thus high levels of GPx1 is likely to reflect high levels of free radicals (i.e. NO) and ROS i.e. oxidative and nitrosative stress (Doherty 2011). It might be appropriate to make the notion than in most studies, and those cited herein, it is not actually the levels of NO that have been measured but the levels of expression of the enzyme nitric oxide synthase (NOS) where the neuronal form (nNOS) is constitutively expressed while the inducible form (iNOS) is expressed during pathological conditions (Doherty 2011; Lubos et al. 2011).

We suggest that the GPx1 expression contributed to the bystander effect we demonstrate by the adult SVZ NPCs. In addition to this, the VEGF expression in these cells most likely had an impact on motor neuron survival by a neuroprotective effect.

On the neuron VEGF is shown to promote axonal outgrowth (Sondell et al. 2000). Furthermore, it is also well documented that VEGF has a neuroprotective effect on motor neuron degeneration in motor neuron disease (Azzouz et al. 2004; Xu et al. 2006; Tolosa et al. 2008). And, in addition, VEGF is expressed in axotomized motor neurons and found to provide neurotrophic support as an increase in the survival of motor neurons has been observed (Skold et al. 2000; Skold et al. 2004; Pereira Lopes et al. 2011).

The VEGF family of proteins and their receptors has been shown not only to be important in motor neuron survival, but they are also found to be intimately involved in regulation and promotion of neurogenesis and are part of the unique neurogenic microenvironments, niches (see introduction) (Jin et al. 2002; Carmeliet 2008; Riquelme et al. 2008; Ekdahl 2012).

Motor neurons respond also to other growth- and neurotrophic factors such as neural growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived factor (GDNF). Both BDNF and GDNF have been found to prevent motor neuron cell loss after ventral root avulsions, and GDNF also to promote axonal outgrowth from motor neurons (Novikov et al. 1995; Tuszynski et al. 1996; Novikov et al. 1997; Wu et al. 2003; Llado et al. 2004; Rodrigues Hell et al. 2009; Su et al. 2009).

Importantly NPCs have been found to express growth factors such as NGF, BDNF and GDNF (Lu et al. 2003; Llado et al. 2004; Su et al. 2009; Foret et al. 2010).

Other growth factors are also expressed by, and involved in the modulation of, NPCs and for example platelet-derived growth factor PDGF is reported to be of importance in the regulation of neurogenesis (Erlandsson et al. 2006; Ishii et al. 2008).

In our experiments we show that NPCs from the lateral ventricle wall of adult rodents express VEGF both *in-vitro*, and after transplantation to the nucl.XII.

4 ACTIVATION OF THE PROGENITOR CELLS

There are some conflicting results on NPC response to the n.XII avulsion injuries in our study. The ependymal region NPCs did not differentiate into neurons and did not have a neuroprotective effect on avulsed motor neurons. One would expect the same signaling pathways to be active in the nucl.XII in response to equipotent injuries.

Therefore, the signals that induced neurogenesis and integration of SVZ NPCs should also be present and available for the endogenous ependymal layer NPCs.

One explanation for this difference could be differences in cell characteristics and responsiveness to the available signals and cues.

As discussed above on endogenous ependymal NPCs in the central canal most report on gliogenic differentiation after injury or disease, without formation of cells expressing neuronal markers (Johansson et al. 1999; Brundin et al. 2003; Mothe and Tator 2005; Meletis et al. 2008; Cizkova et al. 2009; Foret et al. 2010). But, as mentioned, they do possess neurogenic capabilities as neurogenesis from these cells has been reported in EAE and also after spinal cord injury when growth factors were added intrathecally (Kojima and Tator 2002; Danilov et al. 2006). In addition, cells from caudal levels i.e. spinal cord have the ability to differentiate into neurons *in-vitro* and upon transplantation to known neurogenic regions (Weiss et al. 1996; Shihabuddin et al. 2000). Although, in these reports it was not specified from exactly which niche in this part of the CNS the progenitor cells came from. Furthermore, the microenvironment created in the nucl.XII after avulsion apparently contained signals that induced the adult SVZ NPCs to form neurons, integrate and protect.

Except for maybe a gliogenic nature of the ependymal NPCs in the brainstem, or that there were actual differences in the niche between the experiments, other plausible explanations for our findings exist.

First, the survival time was longer in the transplantation experiments (3 months as compared to 28 days) and if extended in time it is possible that we would have found neuronal markers also on cells that originated from the endogenous NPCs. Also, there are fundamental differences in the constitutions of these two regions. The cell composition in the SVZ of the lateral ventricle wall is heterogeneous, including both quiescent and more rapidly dividing progenitors or precursors and here a continuous ongoing neurogenesis prevails (Ihrle and Alvarez-Buylla 2011).

In the central canal and fourth ventricle region it is the ependymal cell that is the NPC, which is considered quiescent. It divides slowly and only in order to maintain the NPC population, thus not giving rise to differentiated progeny during normal physiological conditions (Meletis et al. 2008; Cizkova et al. 2009; Barnabe-Heider et

al. 2010; Foret et al. 2010). Although the ependymal cell of the forebrain SVZ is also considered quiescent during non-pathological conditions, this region contains other proliferative cells (Coskun et al. 2008; Carlen et al. 2009). And, as we did not in detail characterize the cells we harvested from the lateral ventricle wall the NPCs we grafted could very well be ependymal cells, SVZ type B astrocytes and/or the type C cells. Furthermore, the cells we transplanted were contained in cell culture medium, including growth factors as described, and this could also have had a beneficial effect on the transplanted SVZ NPCs favoring survival, differentiation and integration. Also, there were differences in animal strains which could explain differences in the niche in the nucl.XII after avulsion and hence different signals or different levels of signals. It is well documented that variations in genetic factors in the same species but different strains can influence e.g. inflammatory levels after injury with effects on outcome (Popovich et al. 1997; Sroga et al. 2003; Swanberg et al. 2006; Piehl et al. 2007).

Nonetheless, despite these differences and possible explanations for them, both endogenous and transplanted NPCs were activated after a severe motor nerve injury i.e. avulsion but not after transection. And, if now, which could be considered efficient, activation of NPCs is related to the level of injury and hence the assumable need for induction of NPCs in order to repair and replace neurons and other components lost, what are the actual signals involved?

The neural stem or progenitor cells in the neurogenic regions in the adult CNS are in a microenvironment, or niche, where the milieu created by the NPCs and surrounding cells (i.e. astrocytes, ependymal cells, interneurons, microglia, oligodendroglia, perivascular cells, fibroblasts) and components such as diffusible signals and molecular factors in intrinsic and extrinsic signal pathways, extracellular matrix proteins, gap-junctions, basal lamina and the vasculature, where angiogenesis and neurogenesis for example share some factors (e.g. VEGF (Jin et al. 2002)), constitutes a permissive setting which continuously allows for and regulates neurogenesis thus determining NPC fate (Gritti and Bonfanti 2007; Riquelme et al. 2008; Ihrie and Alvarez-Buylla 2011; Morrens et al. 2012).

Many of the signals involved in regulation and activation in adult NPCs are as stated of course also found during development where several of them have multiple or at least dual functions.

Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) are two of the most established mitogens with regards to propagation and proliferation of adult NPCs, not only under cell culture conditions but also *in-vivo* as the EGF receptor has been found in the SVZ and infusion of both of these two mitogens shown to promote proliferation of NPCs (Reynolds and Weiss 1992; Morshead et al. 1994; Craig et al. 1996; Reynolds and Weiss 1996; Kuhn et al. 1997; Kojima and Tator 2002; Martens et al. 2002).

In addition there are a number of important signals and signaling pathways in the regulation of NPCs. The Notch signaling pathway is involved in keeping cells in a stem or progenitor cell state thus inhibiting neurogenesis (Stump et al. 2002). The basic helix loop helix (bHLH) family of proteins can either promote neurogenesis or be inhibitory and mediate self-renewal or induce astrogliogenesis (Kageyama et al. 2005). Furthermore, the Janus kinase (JAK) / Signal transducer and activator of Transcription 3 (STAT3) pathway, where LIF and Ciliary neurotrophic factor (CNTF) are two of the most important signaling molecules, is found to be of importance in the self-renewal of NPCs and neurogenesis in the adult, while downregulation of STAT3 blocks neurogenesis (Quadrato and Di Giovanni 2012). Also of major importance, as extensively discussed previously, are the Sox proteins and the Shh and BMP signaling systems.

How injury and disease affect these factors and signaling pathways has not to date been fully characterized, and we did not in detail examine which factors that on a molecular level are involved in the activation of the endogenous and grafted NPCs. However, the level of motor neuron cell loss was strongly correlated to the activation of both the endogenous NPC pool and the transplanted SVZ NPCs with regards to proliferation, migration, differentiation and integration.

We confirm previous findings, and differences, with regards to motor neuron cell loss but also to the inflammatory and glia cell reactions seen after transection and avulsion injuries (Svensson and Aldskogius 1993; Koliatsos et al. 1994; Yu 1997; Itoh et al. 1999; West 1999; Jiang et al. 2000; Hoang et al. 2003).

These observations were consistent throughout the study with regards to levels of motor neuron cell loss, which was substantially higher after avulsion injuries as compared to the transections. However, we did not evaluate levels of e.g. microglia and macrophages in the transplantation experiments and only assume that the levels of inflammation were equal to the levels and differences found in the endogenous NPC experiments. Importantly though, there are differences with regards to the inflammatory response between the two different animal strains used, the Sprague-Dawley and the Lewis, therefore such conclusions has to come with precautions (Popovich et al. 1997). However, important to notice is that the transplantation experiments were primarily compared to each other, and here the same wild-type and transgenic animal strains were used.

Also, we did detect differences in the levels of the antioxidant enzyme GPx1 in the nucl.XII in the transplantation papers, indicating differences with regards to inflammation between avulsion and transection also in these experiments (Lubos et al. 2011).

We suggest that both motor neuron cell death and neuroinflammation creates a microenvironment, or niche, in the nucl.XII of which the constituents and/or the levels differ between avulsion and transection injury. And therefore the NPC response we report on differ in-between the two types of axotomies.

Thus, it is plausible to discuss on mechanisms for activation with reference also to microglia and inflammation.

Interestingly, microglia and NO are found to be involved in the regulation of neurogenesis in the adult CNS and are shown to affect various signaling pathways regulating NPC self-renewal, proliferation, differentiation and integration (Contestabile and Ciani 2004; Ekdahl 2012). However, it appears that there are dichotomies in regards to the effects as for example neurogenesis can be both promoted and inhibited, it also appears as that the influence differs whether the conditions are normal or during inflammation (Ekdahl et al. 2009; Carreira et al. 2012; Ekdahl 2012).

It has been shown that high levels of NO decreases neurogenesis and promotes gliogenesis but also inhibits proliferation of NPCs of the SVZ, and that blocking of NO synthesis results in an increase of neurogenesis (Covacu et al. 2006; Romero-Grimaldi et al. 2006; Torroglosa et al. 2007). It appears that during normal conditions NO blocks neurogenesis, keeping the NPCs in a progenitor cell state, but during injury and inflammation exerts a neurogenic effect on them - however with differences that are dependent on the type and severity of the injury (Carreira et al. 2012).

Microglia is found to have some detrimental effects on neurogenesis (Monje et al. 2003; Iosif et al. 2006). However, the dual role of microglia in adult neurogenesis is becoming more evident, and microglia have been found to exert beneficial effects on NPC survival and proliferation, for example by chemokine signaling between neurons and microglia, and by production of growth factors (Iosif et al. 2006; Ziv and Schwartz 2008; Bachstetter et al. 2011; Ekdahl 2012).

5 PROGENITOR CELLS IN THE FILUM TERMINALE

The rationale and background for these experiments were the findings on both adult SVZ NPC grafting and the insufficient response in the endogenous NPCs after nerve avulsion injuries.

Therefore the development of an autologous transplantation model was initiated.

In order for this to be possible additional experimental work was needed and several criteria had to be fulfilled. First, we needed a potential tissue candidate where cells with NPC characteristics exist. Secondly, tissue from such an anatomical site would have to be readily accessible without too large risks associated with harvesting. Furthermore the tissue should not only contain NPCs, but cells from here would have to be possible to propagate *in-vitro*.

Obviously also, the cells have to be able to survive grafting, differentiate and integrate in the injured region and/or produce factors favorable for regeneration and functional improvement.

However, initially the first three criteria had to be elaborated on. With regards to these our lab has previously shown that it is possible to harvest SVZ NPCs from humans during endoscopic neurosurgical procedures (Moe et al. 2005; Westerlund et al. 2005). Although minimally invasive and considered a safe and well established neurosurgical

procedure this procedure involves brain parenchyma to be penetrated by the endoscope, and also, although successfully cultured, the cells from here have turned out to be very difficult to process.

For these reasons we turned into other sources for cell harvesting. Since the central canal, lined by cells with NPC characteristics, has been found to extend caudally into the filum terminale (FT) where also glia cells, neurons and possibly immature neurons are found, we decided on to characterize this structure further (Choi et al. 1992; George et al. 2003; Rethelyi et al. 2004; Fontes et al. 2006).

In addition, cells from the human FT have been demonstrated to possess NPC features as they proliferated, formed neurospheres, self-renewed, expressed NPC markers and upon differentiation developed features characteristic for neurons, oligodendrocytes and astrocytes (Varghese et al. 2009). The cells were also grafted into an animal model of stroke and found to migrate to the lesion site and differentiate into astrocytes. However, no neuronal markers on grafted cells were observed *in-vivo*.

A protocol for harvesting, characterization and culturing of tissue and cells from the human FT was developed.

The tissue was obtained from donors undergoing the neurosurgical procedure of detethering of a tethered spinal cord (Warf 2004; Fontes et al. 2006; Liu et al. 2011). We demonstrate that cells expressing Sox2 and Mushashi-1 are abundant and present throughout the entire length of the FT. Thus, this shows that it is not necessary to identify a certain part of the FT for future therapeutic use, and also that if a reasonable part of it is resected it is likely that cells can be obtained in adequate numbers for grafting. Furthermore, Sox2 and Mushashi-1 expressing cells were not confined to the ependymal layer surrounding the central canal, but also subependymal organized bands and clusters of cells expressing NPC markers were observed. Interestingly, the distribution of NPCs in the FT demonstrated similarities to the findings reported on from human spinal cord, where NPCs are shown to be present also in a subependymal layer (Dromard et al. 2008).

When taken to culture conditions we found that cells from the FT could be propagated and that they differentiated into neurons and astrocytes. The cells were found to behave in a similar manner as the adult SVZ NPCs. Furthermore, clonal expansion indicated a relatively strong self-renewal capacity.

We observed differences in growth capacity as FT from younger donors were easier to expand and showed higher proliferative ability. This is in line with findings on adult NPCs where it is found that neurogenesis is decreased with higher age (Chen et al. 2006; Luo et al. 2006; Encinas et al. 2011). Importantly though, we were able to show that the self-renewing properties were maintained also in tissue from adult older donors (> 30 years of age).

In addition, we show that the FT NPCs respond to PDGF-BB with a significant (almost tenfold) increase in the amount of neuronal cells. PDGF signaling is important for self-renewal and neurogenesis, both in the developing and adult nervous system, and this growth factor family is composed of five hetero- or homodimeric PDGF ligands and

three tyrosine kinase receptors (Fomchenko and Holland 2007). The PDGF-BB is shown to activate all three of the receptor isoforms i.e. $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ (Williams et al. 1997). It appears that the PDGF-BB ligand, together with the PDGF-AA and AB ligands, is primarily important for survival and proliferation of NPCs and immature neurons and not involved in fate determination (Johe et al. 1996; Williams et al. 1997; Erlandsson et al. 2006). Although it has been suggested that PDGF signals are also involved in neuronal induction (Johe et al. 1996).

In the postnatal and adult CNS both the PDGF- α and β receptors have been found to be expressed by NPCs in the SVZ (Jackson et al. 2006; Ishii et al. 2008). Furthermore, the PDGF is considered to be a potent mitogen of oligodendrocyte precursor cells in the SVZ, and it is suggested that PDGF signaling balances neuron and oligodendrocyte production from SVZ NPCs in cooperation with bFGF (Jackson et al. 2006; Fomchenko and Holland 2007; Rao et al. 2009). Importantly, there are some discrepancies on PDGF findings which could reflect differences in growth factor responsiveness during development and in the adult.

However, our findings show that a NPC pool is maintained in the FT in the young and adult, and that cells from here can be induced to increase neuronal cell numbers upon administration of growth factors i.e. PDGF-BB.

Adult NPCs and PDGF signaling is of importance also on discussions regarding tumor formation, and overexpression of PDGF ligands and receptors are involved in the development of tumors in the CNS (Fomchenko and Holland 2007).

The connections between adult neurogenesis and tumors of the CNS are many, both in general conceptual views as well as in more specified details. Within cancer biology the concept of a cancer stem cell (CSC) has evolved recently. One of these models, the stemness phenotype model, proposes that there is a single CSC with different stem or progenitor cell properties depending on the microenvironmental constituents, which are detrimental, for the rise of multiple neural lineage phenotypes responsible for the heterogeneity of cells within a tumour (Cruz et al. 2012).

This way of looking upon primary brain tumors is very much like the neurogenic niche concept of adult neurogenesis. Furthermore, many of the markers used for identifying NPCs are expressed by glial brain tumors e.g. Sox2, Nestin, Mushashi-1 and CD133 and it is suggested that at least the glioblastoma multiforme has its origin in oncogenic transformation of endogenous NPCs in the SVZ and changes in this niche. For example as discussed above by alterations in growth factor expression, receptors and signaling pathways (Quinones-Hinojosa and Chaichana 2007; Germano et al. 2010).

Also ependymomas and medulloblastomas have been found to contain CSCs which exhibit NPC characteristics involving the same signaling pathways for self-renewal and differentiation, including neuronal differentiation, giving rise to the heterogeneous population of cells also in these tumors (Andreiucolo et al. 2010; Aizawa et al. 2011; Manoranjan et al. 2012).

Thus conducting experiments which involve comparing the NPCs and the CSCs may very well be one strategy to resolve some of the enigmatic features of both adult neurogenesis as well as brain tumor formation – perhaps resulting in the provision of new therapeutic instruments.

6 TREATMENT STRATEGIES AFTER AVULSION INJURIES

A ventral nerve or root avulsion injury comes, as shown, with massive loss of motor neurons leading to devastating functional deficits (Koliatsos et al. 1994; Carlstedt et al. 1995; Hoang et al. 2003; Havton and Carlstedt 2009; Carlstedt 2011). Furthermore, ventral root avulsion injury induces loss of autonomic neurons and intramedullary degeneration of sensory afferent axons and neuropathic pain (Hoang et al. 2003; Bigbee et al. 2007; Bigbee et al. 2008).

Ventral root avulsion injuries in patients are most frequent in the cervical region, but occur also in the lumbar part, and are in the majority of cases the result of high energy trauma e.g. brachial plexus injuries after motor vehicle accidents (Narakas 1985; Havton and Carlstedt 2009). However, also complications during delivery are a major cause.

Approximately 70% will have multiple roots avulsed and as many are likely to experience persisting pain, especially when there is affection of lower cervical roots (Narakas 1985).

6.1 Endogenous Neural Progenitor Cell Activation

The endogenous NPCs, although activated, did not differentiate into neurons. Neither did they appear to exert neuroprotective effects on the motor neurons in the nucl.XII. This does not per se exclude them from a possible fundamental role in motor neuron survival, and adult NPCs from the ependymal layer have been found to express BDNF after for example spinal cord injury (Foret et al. 2010). It could be that they do have the same nursing role as transplanted adult SVZ NPCs, meaning neuronal loss after avulsion would be even more substantial if they did not exist.

This is however speculative with regards to our study, since we did not perform experiments on growth factor or scavenger production, neither were experiments where the NPCs in the ependyma were depleted and thereafter the nerve injured performed. Addition of growth factors could be one way to modulate the endogenous NPC response, in order for them to be candidates in treatments for injury and disease in the nervous system. There are reports of effects on endogenous NPCs in the central canal and fourth ventricle, and also in lesioned spinal cord after administration of growth factors (Kojima and Tator 2002; Martens et al. 2002; Ohori et al. 2006).

The use of endogenous NPCs is an, in theory at least, attractive alternative to transplantations since many of the negative components of grafting do not apply. For example ethical issues with regards to the potential tissue for stem/progenitor cell harvesting, immunological considerations with graft-versus host reactions, the risk of inducing invasive malignancies i.e. tumour formation and of course the risks associated with the invasive procedure of grafting.

6.2 Grafting of Adult Neural Progenitor Cells

The beneficial neuroprotective effects of transplanting adult NPCs on motor neurons after detrimental nerve injuries cannot be neglected.

There are several reports on this neuroprotective effects by growth factor producing stem or progenitor cells. Importantly though, few report on NPCs and their role in the CNS after peripheral nerve injuries, and even fewer on transplantations of adult NPCs in these.

Su and co-workers reported on a neuroprotective effect on motor neuron survival after grafting of NPCs post avulsion, where the effect was attributable to the production of growth factors from the NPCs (Su et al. 2009). They too, failed to demonstrate axonal growth from transplanted eGFP NPCs although they observe that grafts promote host axonal regeneration. The neurotropic effect of NPCs on motor neurons they report on is supported by others. Although *in-vitro*, it was found that motor neurons in spinal cord explants grew axons through the white matter to form new ventral root-like structures (Llado et al. 2004).

However, and important, the transplanted cells in the Su paper were embryonal NPCs – not adult cells.

Furthermore, mesenchymal stem cells from adult animals were grafted to the ventral horns after ventral root avulsion and demonstrated to have a neuroprotective effect similar to what we and the Su group report on (Rodrigues Hell et al. 2009).

As far as I know, our study is the first report on the use of adult SVZ NPCs.

Thus, transplantation of different types of stem or progenitor cells after motor nerve injuries resulting in massive motor neuron loss, i.e. nerve avulsion, has significant beneficial effects on motor neuron survival. Then the question is what cells to be used in future transplantations to our patients?

When it comes to transplantations to the nervous system in general there is a plethora of experimental models, but also some clinical trials, which differs with regards to several factors (De Feo et al. 2012). These factors involve the injury or disease model used, the species and age of the host, whether the transplantation model used included allografting or xenografting and if a targeted direct approach was used with implantation in the region of cell loss, or if cells were grafted into other regions or even injected into the circulatory system demanding migration (Cummings et al. 2005; Parr et al. 2007; Lu et al. 2012; Lundberg et al. 2012; Riley et al. 2012).

Furthermore, several different cells and cell lines have been used, both from human and animals, and also from different locations such as brain and spinal cord (Cummings et al. 2005; Lepore et al. 2006; Parr et al. 2007; Lu et al. 2012; Muneton-Gomez et al. 2012; Riley et al. 2012). In addition, genetically engineered cells and cells primed for replacement of a specific cell type have been used (Bjorklund et al. 2003; Lu et al. 2003).

In most reports on grafting of NPCs embryonic or fetal cells have been used, but it is also suggested that cells from the adult CNS can be used in transplantations, both from

the brain and spinal cord (Pluchino et al. 2003; Westerlund et al. 2005; Parr et al. 2007; Parr et al. 2008).

All of these transplantation strategies bring some obstacles that need to be addressed if they are to be used in the everyday clinical practice. A major limiting factor with embryonal or fetal tissue is tissue availability. But with such tissue obviously also ethical questions and the risk of infiltrative tumor formation, when using undifferentiated tissue with multi lineage differentiation potential, are of importance (Brederlau et al. 2006; Guillaume and Zhang 2008).

In addition, as long as non-self tissue is grafted there is a risk of graft rejection which demands for immunosuppressant medication with several potential side effects, besides the obvious risk of losing all or the majority of grafted cells from a graft vs. host reaction (Rezzani 2006; Guillaume and Zhang 2008; Laguna Goya et al. 2011).

To overcome some of the issues stressed above, for example availability and ethical considerations, mesenchymal stem cells have been used (Parr et al. 2007; Parr et al. 2008; Rodrigues Hell et al. 2009). Although these cells might not be as promising as once suggested, beneficial effects are reported (Barnabe-Heider and Frisen 2008; Rodrigues Hell et al. 2009).

However, taken together and despite the above discussed concerns cell transplantation strategies seem more promising than the use of endogenous NPCs.

As we demonstrate abundance of growth factor responding NPCs in the human FT, also from older donors, we suggest that cells from here be used in autologous transplantations for nervous system repair. Such a repair model would succumb several of the issues raised on cell grafting. And, importantly, it is possible at least from a technical and logistical point of view to apply it to the clinical setting.

6.3 Root and Nerve Implantations

Reimplantation and implantation of peripheral nerve grafts for treatment of avulsion injuries is shown to have beneficial effects on motor neuronal and axonal regeneration in experiments (Carlstedt et al. 1993; Wu et al. 1994; Hoang et al. 2006; Su et al. 2009; Pinter et al. 2010). Interestingly, it seems that the beneficial effects after a root reimplantation is not confined to the one level of implantation, but effects are also observed on adjacent levels (Hoang and Havton 2006). In addition, experiments demonstrate that root reimplantation can improve the allodynia and sensory axon degeneration (Bigbee et al. 2007; Bigbee et al. 2008).

The axonal regeneration from the CNS to the PNS is, at least partially, believed to be due to proliferating Schwann cells, which are associated with the implanted root or peripheral nerve graft and provide trophic support (Li and Raisman 1994; Wu et al. 1994; Ide 1996; Cullheim et al. 1999; Chu et al. 2008; Chu and Wu 2009; Carlstedt 2011).

6.4 Combinational Therapies

As experimental root reimplantation, or nerve graft implantation, is found to have effects on the above, it is most definitely plausible to use this repair strategy in avulsion injuries. It has also been taken to the patients, and is a well established treatment of brachial plexus injuries with root avulsions (Carlstedt et al. 1995; Carlstedt et al. 2004; Havton and Carlstedt 2009; Carlstedt 2011). Although some functional improvement has been observed, additional treatments are needed in order to improve outcome. One strategy is to use a combined approach including both NPCs and root/nerve graft implantation. It has been found that after a ventral root avulsion both grafting of NPCs and implantation of a peripheral nerve graft have beneficial effects on motor neuron survival (Su et al. 2009). The neuroprotective effect was equal in both the group that received NPCs and the peripheral nerve graft group, without any significant differences in motor neuron survival and a combination of the strategies did not improve survival further. Thus no synergistic effect on motor neuron survival could be demonstrated. Importantly though, the combination of transplanted NPCs and nerve grafting promoted host axonal growth. Thus both neurotrophic and neurotropic effects were found and suggests a combinational strategy including NPC and root/nerve grafting. This has to be further evaluated in experiments.

6.5 Timing of Treatment

If to be taken to the patient, the timing of transplantation after motor nerve and root avulsion injuries is of importance.

We found a beneficial effect on motor neuron survival in this study when transplanting the adult NPCs at nine days after avulsion. This was chosen as a time point not too late with regards to the progressive loss of motor neurons after an avulsion, and not too early as we reasoned that the environment immediately after injury would be less permissive for NPC survival and integration.

On the other hand, transplantation at early stages after injury could in fact be more favorable by providing conditions more permissive for graft integration, and also for achieving early neuroprotective effects. However, when transferring ours and others experimental data to a clinical setting, nine to fourteen days is a clinically relevant time point. Clinically relevant because as approximated 70% of patients with severe root or avulsion injuries have multiple associated injuries (Narakas 1985). These are often life threatening conditions that demands imperative treatments, and the patient to be stabilized, before eventually a nerve avulsion injury can be treated. Furthermore, there is time needed for proper and accurate diagnostics of root avulsion injuries including both neuroradiology as well as neurophysiological investigations. And, importantly, cells for grafting must be harvested and expanded in culture before grafting.

The experimental findings on the timing of NPC transplantations support this delayed or subacute regime, not only after motor nerve avulsion injuries, but also for example

when grafting NPCs to spinal cord injuries (Okano et al. 2003; Parr et al. 2007; Lu et al. 2012).

By the above follows also that grafting of cells should not be performed too long after avulsion injury and many of the experimental papers on root reimplantation after root avulsion support an acute reinsertion of the avulsed root due to the progressive motor neuron loss, which after 4 weeks is described to be at levels of 50-80%, well in line with our findings.

Not to transplant in a chronic phase i.e. late is also supported by the findings that clinical restitution of activity after root reimplantation has been observed only in those patients that were operated upon within one month (Carlstedt 1997; Hayton and Carlstedt 2009).

7 CONCLUDING REMARKS

Many of the signals- and signaling pathways involved in regulation and activation of adult neurogenesis are found during developmental neurogenesis. Interestingly, several of these “key players” of regulatory pathways are also involved in the regulatory machinery responsible for the events occurring after nerve injury with regards to motor neuron survival and death, but also with regards to repair after other injuries and diseases in the CNS (Kiryu-Seo and Kiyama 2011; Quadrato and Di Giovanni 2012). This can at least to some part be understood if seen as that the neurons affected change from an active signaling state to a more rudimentary, survival- and growth promoting state.

Thus, in order for repair strategies with neural stem or progenitor cells aiming at regeneration and partial or full restoration of functional deficits in the nervous system, affecting not only motor nerve injuries as investigated in this thesis work, the understanding of these molecular mechanisms are fundamental.

This can only be done by continuing with well designed and thought through experimental studies.

To conclude, there are many issues to be further evaluated and questions to be answered, and although we are not even close to the beginning of the end for the experimental work on adult neurogenesis to be finished, in order for the findings to be brought to the patients, we are closer to the end of the beginning today than we were yesterday.

CONCLUSIONS

I. In response to cell loss by nerve avulsion the ependymal layer cells in the brainstem regenerative zone of the central canal and 4th ventricle proliferated and the progeny cells migrated into the lesion area. The progeny formed astrocytes but no neurons.

II. In this model of transplantation to immunocompetent animals grafted NPCs formed neurons that integrated with host neurons. As a bystander effect, the grafting procedure significantly improved the number of surviving motor neurons.

III. The severe injury (avulsion) was significantly more permissive for survival, differentiation and synaptic integration of grafted NPCs. Cells grafted into the severely injured area up-regulated antioxidant and trophic mechanisms.

IV. Neural progenitor cells are present in the human filum terminale, also in the adult. They are abundant and exist along the entire filum terminale. Similar to the SVZ NPCs they form neurospheres and can be propagated in culture. Upon differentiation they express markers for neurons and glia. Administration of PDGF-BB results in an increase in neuronal cell numbers. Thus this supports their potential use as candidate cells in future transplantation models.

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