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Progenitor cells in the postnatal central nervous system, characterization and genetic manipulation

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Progenitor Cells in the Postnatal Central Nervous System

Characterization and Genetic Manipulation

Akademisk avhandling
Av
Nina Rogelius

Som med vederbörligt tillstånd av medicinska fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i Segerfalksalen, Wallenberg neurocenter, Lund

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Abstract

For decays it was believed that no new neurons were generated in the adult brain. We now know that generation of new neurons takes place in two regions, the subventricular zone and hippocampus. It seem like the brains ability to repair it self and generate new neurons to specific areas where cells are needed e.g. after cell loss from brain disease are limited. However, the cells in the subventricular zone are capable to migrate long distance. With more knowledge about their plasticity it might be possible to direct the new cells to a certain fate and thus potentially serve as a basis for brain repair. Using retroviral vectors we have genetically modified the new cells in the SVZ with the genes Islet1 and Neurogenin2 with the aim to direct them into the striatum. Our results showed that with Isl1 transduction the newborn cells could indeed migrate into the striatum. However, the cells expressed Ng2, a marker for glia progenitor rather than neuronal markers. Interestingly, transduction with Isl1 in combination with Ngn2 also resulted in migration to striatum and in addition also into the external capsule. These cells displayed a neuronal-like morphology and once removed from the striatum to culture dish they also expressed the neuronal marker b-III-tubulin. In conclusion, the results indicate that progenitor cells in the SVZ can be directed by over expression of developmentally important instructive genes and may migrate into the striatum and show potential for neuronal differentiation at this site.

Key words: Progenitor cell, stem cell, neurogenesis, transcription factors, subventricular zone, Islet1, Neurogenin2, respecification, *in vivo*

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Progenitor Cells in the Postnatal Central Nervous System

Characterization and Genetic Manipulation

Nina Rogelius

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Retroviral labeled GFP expressing cells in the rat olfactory bulb originating from the normal postnatal SVZ.

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När du ska ta hand om andra, använd hjärtat.

CONTENTS

Summary

SUMMARY

Most neurogenesis is completed at birth but in the subventricular zone and hippocampus neurogenesis is still maintained in the postnatal and adult mammalian brain including humans. The neural progenitor cells in the SVZ generate proliferating neuroblasts that migrate in the RMS to the OB were the newly derived cells differentiate to interneurons, granule and periglomeruli cells. It is those migrating neuroblasts that the research in this thesis focuses on. We have genetically manipulated the neuroblasts with transcription factors to investigate if this could generate a new migration pattern and differentiation fate. The knowledge of how to direct the endogenous progenitor cells to a certain fate can be of great importance for repair in neurodegenerative disorders, then new cells could be added to the area suffering from cell loss. With the aim to direct the cells to a neuronal fate in striatum we have ectopically expressed Islet1 (Isl1) and Neurogenin2 (Ngn2) in the SVZ progenitor cells. Since, Isl1 and Ngn2 are important for cell fate specification during embryonic development, we hypothesized that ectoptic expression of Isl1 and Ngn2 to the postnatal progenitor cells would generate cells that differentiate into neurons. To obtain over expression of Isl1 and/or Ngn2 we constructed retroviral vectors that expressed the respective genes in combination with the marker gene green fluorescent protein (GFP). Our result showed that ectopic expression of Isl1 and Ngn2 in the postnatal SVZ progenitor cells could direct the progeny to a new fate. With only Isl1 transduction the newborn cells migrated to stiriatum and there they expressed the glial marker Ng2. In conclusion, the results indicate that progenitor cells in the SVZ can be directed by over expression of instructive genes and may migrate into the striatum and show potential for neuronal differentiation at this site.

Aims and results of this thesis

THE AIM OF THIS THESIS

To direct the postnatal SVZ progenitor cells to a new fate and by genetic manipulations with proneural genes instruct them to migrate and differentiate into striatal neurons.

THE RESULT OF THIS THESIS

The results in this thesis have given a deeper understanding and knowledge of the progenitors cells in the SVZ and their potential to be directed by genetic manipulations. Moreover, the data show that progenitors cells in the postnatal SVZ are plastic enough to be instructed by genetic manipulations, leading to a new fate with potential to differentiate into neurons.

Original papers

ORIGINAL PAPERS

I. *In vivo* labeling of neuroblasts in the subventricular zone of rats.
Rogelius Nina, Ericson Cecilia, Lundberg Cecilia.
J of Neuroscience Methods 2005.

II. Retrovirally delivered Islet-1 increases recruitment of ng2 expressing cells from the postnatal SVZ into the striatum.
Rogelius Nina, Jensen B Josephine, Lundberg Cecilia, Parmar Malin.
Expreimental Neurology 2006.

III. Expression of Isl1 and Ngn2 in neonatal SVZ results in a striatal cell populatioin with potential for neuronal differentiation.
Rogelius Nina, Jensen B Josephine, Lundberg Cecilia, Parmar Malin.
Submitted.

Introduction

INTRODUCTION

The discovery of postnatal CNS neurogenesis

To day we know that the adult mammalian brain, including the human brain (Eriksson et al., 1998; Curtis et al., 2007), consist of dividing, neural stem cells, with the ability to generate new neurons (Luskin, 1993). By using tritiated thymidine labeling, Altman and Das suggested this existents of neurogenesis in the postnatal hippocampus already in 1965 and a few years later (1969) in the olfactory bulb (OB). However, because of the lack of neuron-specific immunohistochemical markers at that time it wasn't until the 1990-talet that neurogenesis in the postnatal brain started to be accepted (Gage et al., 1995). Now, the endogenous neural stem and progenitor cells are a hot topic for their potential ability to generate new neurons of neurodegenerative disorders, the self-repair (Rossi and Cattaneo, 2002; Lindvall et al., 2004).

Definition of neural stem and progenitor cells

The dividing cells that contribute to the generation of new cells are often termed stem cells. A general definition of a stem cell is a proliferative cell both capable of giving rise to either more stem cells (self-renew) and/or to new differentiated cells (Gage, 2000). This definition is generally accepted of most researchers. The most primitive and potent stem cell is totipotent, by that means that the cell is able to generate all the cell types and tissues that make up an embryo. Embryonic stem cells are pluripotent, able to give rise to every cell of the organism (all three germ layers), but not extra embryonic tissue such as the placenta. Cells with more restricted potency are considered as multipotent and generate only cells of the organ where they originate (McKay, 1997). The neural stem cells are multipotent and can self-renew as well as generate all neural cell lineages of the CNS: neuron, astrocytes and oligodendrocytes (Taupin and Gage, 2002; Seaberg and van der Kooy, 2003). If a cells differentiation or proliferation potential is limited the term progenitor cell can be used. Progenitor cells are the term used in this thesis for the most form of multipotent or lineage-restricted mitotic cells (Kessler et al 2001).

The embryonic proliferative zone.

During development, neurons are generated at early embryonic stages followed later by the generation of glia cells. The two proliferative zones that during development generate the different cell types in the forebrain are the ventricular and subventricular zones (VZ and SVZ, respectively) (Sturrock and Smart, 1980). The VZ is lining the ventricles and consists of proliferating cells which can divide either symmetrically giving rise to two new progenitor cells or asymmetrically and generate a progenitor cell and a postmitotic cell that will differentiate into a neuron or a

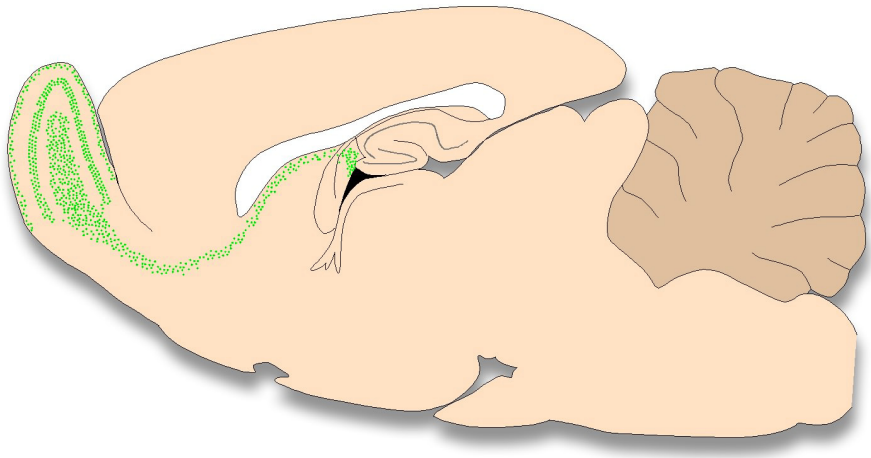

Figure 1.
The endogenous progenitor cells in the SVZ and their migration in the RMS to the OB.

glia cell. Additionally, the VZ proliferative cells give rise to a secondary proliferative zone, the SVZ, which lies adjacent to the VZ (McKay, 2000; Kintner, 2002). In the ventral forebrain the proliferative cells of the SVZ that express the transcription factor Islet1 contribute to the development of striatal projection neurons (Stenman et al., 2003). Progenitor cells in the VZ express Ngn2, which participate in specification of neuronal phenotypes (Fode et al., 2000). The exact origin of the postnatal and adult SVZ and its relation to the embryonic proliferative zones are not fully understood, but the remnants of these embryonic regions of proliferation (VZ/SVZ) are at birth composed of ependymal cells and subependymal layer. This is one of the zones that neurogenesis persists in the postnatal brain.

The location of the postnatal neural progenitor cells.

There are two regions were neurogenesis constitutively occurs in the mammalian postnatal brain, the SVZ of the lateral ventricular walls and the dentate gyrus in the hippocampus. The progeny from the precursor cells in the SVZ migrate in the rostral migratory stream (RMS) and differentiate into granule or periglomerular interneurons of the OB(Luskin, 1993; Doetsch et al., 1999) (figure 1). In the dentate gyrus cells from the subgranular zone generate the hippocampal granular neurons (Kempermann and Gage, 2000). These two regions are generally accepted as neurogenic regions. In addition to these regions, there are single reports of neurogenesis in other regions of the brain such as neocortex (Gould et al., 2001), amygdala (Bernier et al.,

2002), CA1 in the hippocampus (Rietze et al., 2000), brainstem (Bauer et al., 2005), the spinal cord (Yamamoto et al., 2001), and the substanstia nigra (Zhao et al., 2003). However, these results are contradictory to other published reports or have not been reproducible by other groups within the field (Horner et al., 2000; Lie et al., 2002; Koketsu et al., 2003; Frielingsdorf et al., 2004).

The migration of the postnatal neural progenitor cells

The new neurons in the OB have as mentioned above migrated from the SVZ through the RMS to the OB. Specialized astrocytes form a tubular structure, on which the neuroblasts close to each other migrate anterior to the OB, a process called chain migration (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996). Less than a week after the cells have left the SVZ the neuroblasts reach the OB in the neonatal rodent. In the adult migration from the SVZ to the OB all the way through the RMS takes approximately 1-2 weeks (Carleton et al., 2003; Rogelius et al., 2005). Migration can be classified as radial or tangential depending on the orientation of the cell movement in relation to the pial surface. Upon reaching the OB, neuroblasts switch from tangential to radial migration and invade the OB parenchyma, where they complete their differentiation into either granular or periglomerular neurons (Luskin, 1993; Alvarez-Buylla and Garcia-Verdugo, 2002). Also in humans migration of neuroblasts to the OB occurs, it has been suggested that progenitor cells migrate in chains, as in rodents (Curtis et al., 2007).

The identity of the postnatal neural progenitor cells

Doetsch et al 1999 suggests that the identity of the neural stem cells in the SVZ is the single ciliated astrocyte-like cell. Also the multiciliated ependymal cells have by another research group been suggested as the stem cells that give rise to new neurons in the adult CNS (Johansson et al., 1999). Even the mature neurons in the adult brain have been suggested to divide (Brewer, 1999; Gu et al., 2000). To day the generally accepted view is the astrocyte-like hypothesis (Doetsch et al., 1999; Laywell et al., 2000). The astrocyte-like stem cells in the SVZ give rise to rapidly proliferating cells, which in turn generate the migrating neuroblasts that travel through the RMS to the OB. Astrocytes outside of the SVZ and subgranular zone in hippocampus do not appear to be neurogenic in vivo under normal conditions.

Postnatal and adult neurogenesis

Adult neurogenesis has been studied intensively in rodents (Luskin, 1993; Lois and Alvarez-Buylla, 1994) but also some studies in adult primates exist (Gould et al., 2001) including human (Eriksson et al., 1998; Curtis et al., 2007). In the adult rodent brain, neurogenesis occurs in the SVZ and hippocampus throughout life. 9000 new

cells are born every day in dentate gyrus (Kempermann et al., 1997; Cameron and McKay, 2001) and the number is even higher in the SVZ (Zhao et al., 2003), however most of the newly born cells die before they mature into functional neurons (Winner et al., 2002). It has been estimated that 30 000 new OB neurons are generated every day. Though, as during the embryonic development, many new neuroblasts in the adult brain are generated but only a subset of them mature and differentiate (Biebl et al., 2000). Neurogenesis is defined as the process from mitosis to neuronal differentiation, maturation, synapse formation, integration into the neuronal circuit, and survival. Thus it is important to bear in mind that generation of immature neuroblasts from stem cells is not equivalent to neurogenesis. It has been shown that even in the adult human brain, some astrocytes lining the lateral ventricle divide in vivo and behave like multipotent neural progenitors in vitro (Sanai et al., 2004). Curtis et al 2007 demonstrates that human brain also contains RMS where neuroblasts migrate from SVZ to the OB.

Self-repair and Aim

In neurodegenerative disorders, it would be of great importance if the brain its self could generate new neurons that could repopulate the area of neurodegeneration by using its own progenitors cells, thus repairing it self. However, to our knowledge today the ability of the brain for spontaneous self-repair seems limited. However, it is possible that with stimulation through appropriate instructions or addition of the right signals the progenitor cells they might be directed to a new fate. Indeed, recent studies have shown that postnatal production of striatal cells can occur under experimental conditions such as stroke and BDNF administration (Benraiss et al., 2001; Arvidsson et al., 2002; Chmielnicki et al., 2004), suggesting that a precursor with a latent potential for striatal neuron differentiation exists in the SVZ after birth.

The aim of this thesis is, by genetic manipulation of the progenitor cells direct them to a new fate. We have used the developmentally important transcription factors Islet1 (Isl1) and Neurogenin 2 (Ngn2) to genetically modify the progenitor cells. These two factors are important for the generation and specification of neurons during the embryonic development, but in the postnatal brain they are to a large extent down regulated. We proposed that reintroduction of Isl1 and Ngn2 to the postnatal SVZ cells might be the right instructions for the cells to differentiate into stratial neurons. Here we show that despite not being sufficient to instruct the postnatal SVZ cells to become striatal projection neurons, Ngn2 and Isl1 in combination recruited cells to the striatum and upon in vitro differentiation showed potential to generate neurons.

Results and general discussion

RESULT AND GENERAL DISCUSSION

LABELING OF SVZ PROGENITOR CELLS (paper I)

How to study neurogenesis

In the first study of this thesis we studied the dividing cells in the postnatal SVZ and followed their fate. To be able to do this the dividing cells, the neuroblasts, need to be labeled. One way to label the cells that we and other research groups (Carleton et al., 2003; Magavi et al., 2005) have used is viral vectors, which insert their own genome into the chromosomes of the cell they infect and thereby genetically mark the cells permanently. The integrated marker gene (e.g. GFP) carried by the vector is transferred to the progeny of the cells that were originally infected. Retroviral vector integration occurs only to cells in S-phase, thus the genetically labeling is limited to dividing cells. After initial cell infection the viral vector is not spread because the vector it self is incapable of making proteins required for retrovirus replication (Miller et al., 1990; Lewis and Emerman, 1994). Thus, retroviral vectors can infect the dividing cells at the injection time but cannot replicate further. To enlarge the viral vector range of infected cell types the glycoproteins from the vesicular somatitis virus (VSV-G) is used to build up the envelope of the vectors. The VSV-G pseudotyped virus vector is not dependent on a recognition site on the cell surface but interacts with the hosts phospholipids and enters the cell by membrane fusion. The visualization of cells after retroviral labelling allow for complete morphological analyzes.

Retroviral vector injections label the progenitor cells in the neonatal SVZ

We have injected retroviral vectors encoding GFP (Rv.GFP) to the lateral ventricle of neonatal animals at postnatal day one. The vector-transduced cells could in the microscope be visualized as green GFP expressing cells. This injection resulted in transduced cells in the SVZ two days after injections. Thus showing the presence of mitotic active cells in this area (figure 2). The cells in the SVZ contributing to neurogenesis have by others been characterized and identified as astrocyte-like stem cells (called type B), fast dividing progenitor cells (called type C), and migrating neuroblasts (called type A) (Doetsch et al., 1997). The astrocyte-like cells are slowly dividing (Morshead et al., 1994) and express markers such as GFAP and vimentin but don't express PSA-NCAM and b-III-tubulin. This astrocytic-like cells have irregular shape, reside in the SVZ and send out a single ciliated process touching the ventricular surface (Doetsch et al., 1999; Garcia et al., 2004). Cell division of these GFAP positive stem cells generates the fast dividing progenitor cells (Doetsch et al., 1999; Capela and Temple, 2002; Garcia et al., 2004). They are the most actively proliferating cells in the SVZ, large in size, have immature morphology, make up 10%

of the SVZ cells, and are only rarely found in the RMS. They express Dlx2 and nestin but not GFAP, vimentin, PSA-NCAM, or b-III-tubulin. These fast dividing cells give rise to migrating neuroblasts. The neuroblasts are negative for GFAP and vimentin but express nestin, PSA-NCAM, DCX, b-III-tubulin, and Dlx2. They have elongated cell bodies and few processes. It is these migrating neuroblasts that migrate in the chain migrating process through the RMS (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996). In this periventricular region closest to the lateral ventricles reside the ependymal cells. They are multicilated and express vimentin, nestin, and low level of GFAP (Doetsch et al., 1997). Moreover, the SVZ, also consists of Ng2 expressing progenitor cells they generate interneurons and oligodendrocytes in the postnatal OB (Aguirre and Gallo, 2004).

In our study a small fraction of the GFP expressing cells in SVZ were co-labeled with GFAP (Rogelius et al., 2005) indicating that a few astrocytic-like stem cells were transduced and labeled by retroviral injections. The fact that we only detect a few GFP and GFAP double-labeled cells is in agreement with the notion that astrocytic-like stem cells are slow dividing (Morshead et al., 1994). At six weeks after SVZ transduction no cells could be detected in the SVZ but still in the OB transduced cells could be detected this wash-out of cells indicate that the main part of the cells that we transduce was the fast dividing progenitors (type C) and neuroblasts (type A).

Retroviral vector injections label the progenitor cells in the neonatal RMS and OB.

As described in the introduction the normal way for the progenitor cells from the SVZ is migration to the OB through the RMS (Lois and Alvarez-Buylla, 1994). In our study transduced neuroblasts could be detected in the RMS one week after vector injection. Also at two weeks after injection a lot of GFP expressing cells could be detected in the RMS (figure 2). The first transduced cells reached the OB at one week after injection and after an additional week the newly derived cell had migrated out into the different layers of the OB. While the cells migrate in the RMS they expressed morphology typical of migrating neuroblasts with elongated cell bodies and a leading process. Some of the cells could be detected with DCX, a protein normally expressed by migrating neuroblasts in the SVZ and RMS (Gleeson et al., 1999; Nacher et al., 2001). Shown by others, once reaching the OB the majority of the newly generated cells differentiate into GABAergic granule neurons, and only a small proportion develops into periglomerular neurons with GABAergic and dopaminerigic phenotypes. The mature neuronal marker NeuN is expressed in OB interneurons (periglomerular and granule cells) (Consiglio et al., 2004; Weiler and Benali, 2005; Curtis et al., 2007). The transcription factor Er81 is involved in the generation of OB interneurons and label a subset of periglomerular cells (Stenman et al., 2003). Dopaminerigc neurons, identified by expression of TH are almost exclusively expressed in few glomerular layer neurons (Baker et al., 1983). We could in our study label the transduced cell in

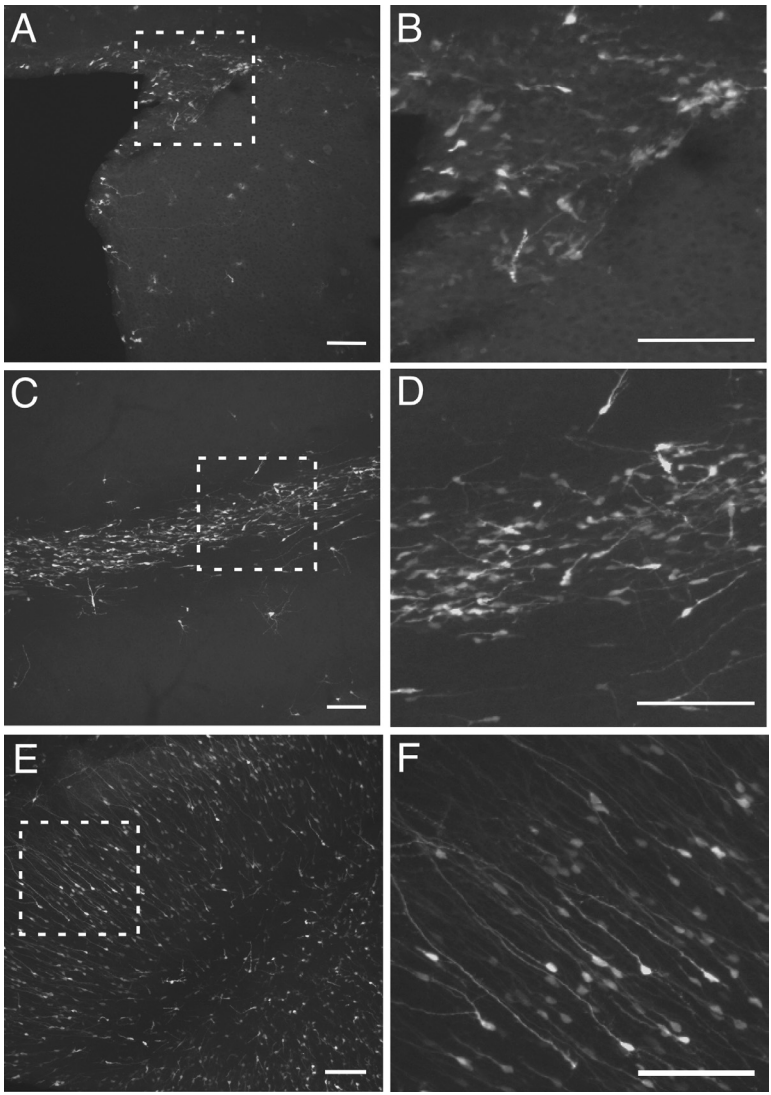

Figure 2.
GFP transduced cells in the neonates.
(A) Labeled cells in the SVZ after injection into the lateral ventricle of a newborn animal at 2 days. (B) Higher magnification of (A) showing the morphology of the cells. (C and D) depict migrating cells in the RMS after 1w. Note the abundance of transduced cells, which in higher magnification (D) show morphologies typical of migrating neuroblasts, including a leading process. In (E) the olfactory bulb is shown, after 3w the GFP positive cells disperse out into the different layers of the structure and present profiles of mature neurons as shown in (F).

the OB with NeuN or Er81, no cells could be double labeled with TH. The remaining periglomerular neurons contain either calretinin or calbindin (Toida et al., 1998). The morphology of the transduced cells in the OB cells in our study was differentiated cells with long processes (Rogelius et al., 2005). The same morphology has been demonstrated in other studies using similar labeling technique (Petreanu and Alvarez-Buylla, 2002; Yamada et al., 2004). Even, six weeks after injection transduced cells could be detected in the OB but no or very few cells could be detected in the SVZ and RMS.

We also injected the retroviral vector directly into the parenchyma of the SVZ instead of into the lateral ventricle and the same pattern of transduced cells in the SVZ, RMS and OB could be detected. Taken together by injection of retroviral vectors into the lateral ventricle or SVZ of neonates label the actively migrating neuroblasts in the SVZ/RMS/OB region. However, due to the small size of the neonatal rat brain injection into the lateral ventricle is favored when aiming for transduction of the SVZ cells only to avoid any risk of infecting nearby striatal parenchyma when injecting into the parenchyma of the SVZ.

Retroviral vector injections label the progenitor cells in the adult SVZ, RMS, and OB

In adults, the SVZ also contain dividing cells. To study them we have injected Rv.GFP into lateral ventricle or the SVZ. As in the neonatal animals transduced cells could be detected in the SVZ, RMS and OB. Similar to the neonatal animals transduced cells in the RMS showed morphology of migrating neuroblasts as described above. However, the number of transduced cells in the adult animals is low. The highest amount of detectable cells was seen two weeks after injections and with vector injections directly into the SVZ parenchyma instead of injections to the lateral ventricles. Even though there is a detectable amount of labeled cells in the adult animals, the number was much less compared with what was seen after neonatal injections. This finding could be due to the fact that the precursor cells in neonatal animals divide more frequently. Indeed, we were able to transduce 10-20 fold more cells with the same vector dose in neonatal compared to adult animals. With the aim to study the actively dividing progenitor cells we mainly used neonatal animals to have the lager pool of proliferating cells to study rather then the rare events of labeled cells in the adult animals.

Summary of paper I

"In vivo labeling of neuroblasts in the subventricular zone of rats"

In this study (paper I), we have shown that the endogenous SVZ progenitors cells and neuroblasts can be labeled and transduced with GFP expressing retrovirus. This enables us to follow their migration and differentiation both in neonates and adults.

Retroviral vector are a promising tool for transduction of the SVZ progenitors with instructive genes to direct the progeny to a new fate. The stem cell is not labeled in sufficient number to study them. The use of lentiviruses transduced dividing cells in the SVZ and also non-dividing and slow dividing cells (Consiglio et al., 2004; Rogelius et al., 2005). To target and focus the study on the actively dividing neuroblasts in the SVZ the use of retroviral vectors was preferred. The limits of retroviruses to transduction of and only specifically label the progenitor cells is an advantage since it keeps the slow-dividing stem cells intact and don't permanently change their genome.

A NEW CELL POPULATION IN STRIATUM (paper II)

In the next study (paper II) we wanted to investigate the possibility to direct the progenitor cells in the postnatal SVZ to a new fate. We studied the role of the transcription factor Isl1 in generation of striatal projections neurons from the SVZ progenitors in neonates and adults. Isl1 is expressed during development in the mitotic progenitor cells of striatal projection neurons (Pakzaban et al., 1993; Deacon et al., 1994; Olsson et al., 1995). Around birth, Isl1 is down-regulated in the SVZ and at the same time the production of striatal projection neurons ceases (Toresson and Campbell, 2001; Wang and Liu, 2001; Stenman et al., 2003). To determine whether Isl1 expression in neonatal SVZ progenitors could affect the phenotype of the cells generated from this area we have reintroduced Isl1 to the SVZ progenitor cells in both neonates and adults by using retroviral vectors coding for Isl1-IRES-GFP (Rv.Isl1). The SVZ cells were infected with either Isl1 (detected by GFP expression) or GFP only as a control.

Ectopic Isl1 expression in neonatal SVZ does not interfere with OB differentiation

Two days after injection, GFP expressing cells could be detected at the injection site in the Rv.Isl1 injected animals. 1-3 weeks after injection extensive migrations of Isl1 transduced cells could be detected in the RMS on their way from the SVZ to the OB in the neonatal animals. The Isl1 transduced cells migrating in the RMS appeared phenotypically normal: the cells displayed morphologies characteristic of migrating neuroblasts and expressed DCX. In the OB, Isl1 transduced cells displayed the location and morphology as in the control animals and both the Isl1 and control transduced cells co-expressed Er81. Thus, in the context of the neonatal environment Isl1 does not seem to disrupt the migration of SVZ-derived neuroblasts in the RMS, or their subsequent differentiation into OB interneurons.

Ectopic Isl1 expression in neonatal SVZ promotes striatal recruitment

Interestingly, Isl1 expression in the neonatal SVZ cells promotes migration of cells to the striatum. This new population of migrating cells could be detected in the ventrolateral part of striatum and was uniquely found in the Isl1 transduced animals, and not found in control animals (figure 3). The phenotypic characterization of the GFP expressing cells in the striatum showed that the cells from two weeks post-injection expressed the glial progenitor marker Ng2 and at six weeks virtually all cells expressed Ng2 (figure 4). The transduced cells recruited to the striatum did not express any of the specific markers of mature glia cells tested (GFAP, S100b or CNPase). Ng2 expressing cells often differentiate into oligodendrocytes in vitro and in vivo (Nishiyama et al., 2002; Dawson et al., 2003; Bu et al., 2004). Some groups

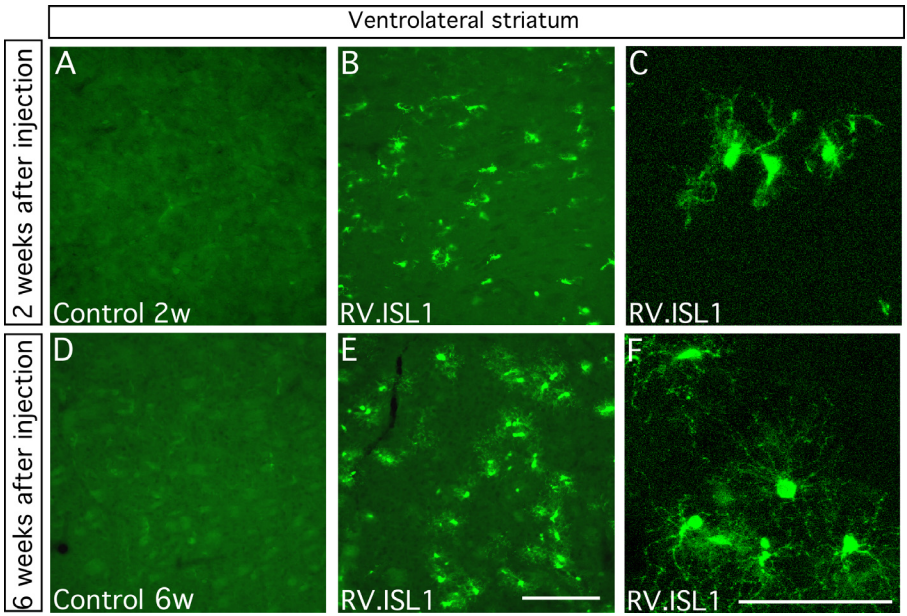

Figure 3.
Ectopic ISL1 expression in neonatal SVZ cells promotes striatal recruitment.
Two weeks after injection of the control vector virtually no GFP positive cells could be detected in the ventrolateral striatum (A). In contrast, in the RV.ISL1 injected group numerous GFP positive cells could be detected in this area at the same survival time point (B). The cells within this novel population displayed immature morphology (C). Six weeks after RV.ISL1 injection, the striatal cell population was still present (E,F) whereas no cells at all could be detected in the control injected animals (D). All images are from the ventrolateral striatum. Scale bar 100μm.

report that Ng2 expressing cells not only generate glia cells but also retain their ability to produce other neural cell types (Aguirre and Gallo, 2004; Sellers and Horner, 2005). The full differentiation potential of the Ng2 expressing cells recruited to the striatum after Isl1 expression remains to be determined. From our results, it is not clear weather the Ng2 expressing cells recruited to the striatum represent a progenitor cell that are arrested in their further development but that harbors a potential to, given the proper differentiation cues, fully differentiate into neurons or other mature cell types reported to be the progeny of Ng2 cells (reviewed in (Sellers and Horner, 2005) or weather they represent terminally differentiated Ng2 expressing glia as described by Peters (2004).

In both groups, GFP expressing cells are found in the SVZ and striatal parenchyma close to the LV (Zone I). In Zone II, located further away from the LV, only few GFP expressing cells could be detected in the control group, but a substantial number

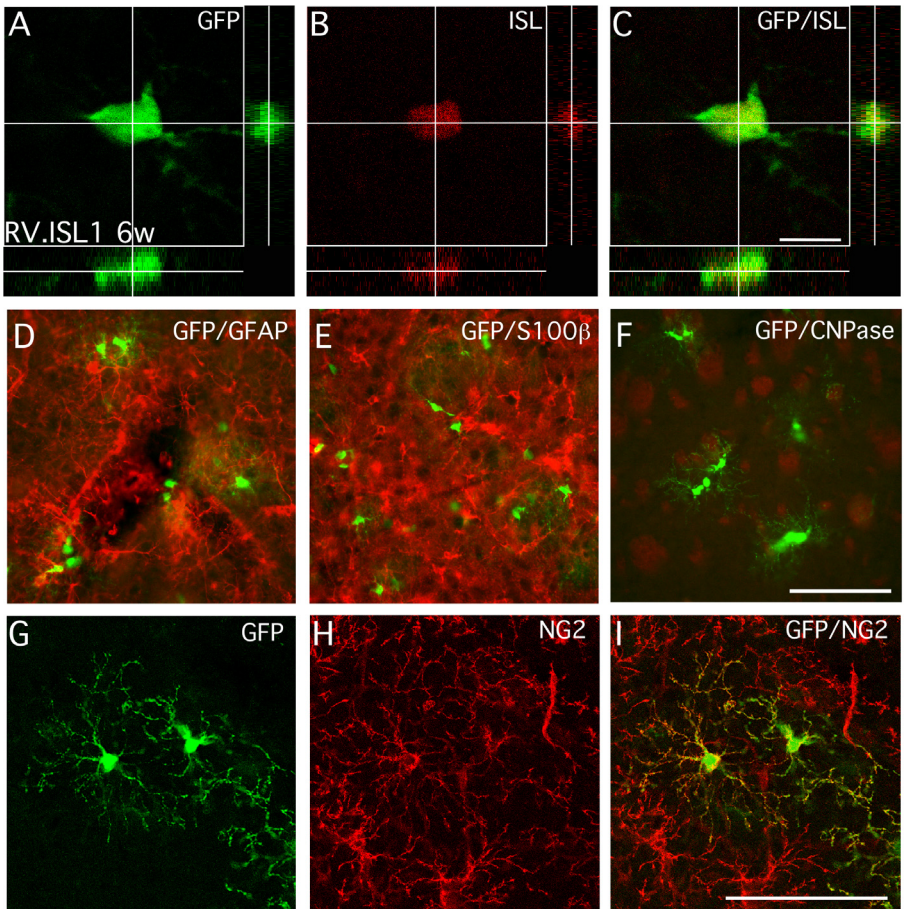

Figure 4.

Phenotypic characterization of the cells recruited to the striatum 6 weeks after RV.ISL injections into the neonatal SVZ.

All of the transduced cells still co-expressed ISL1 and GFP (A-C). The RV.ISL transduced cells in the ventrolateral striatum all had a glia-like morphology, but did not express GFAP (D), S100b (E) or CNPase (F). The great majority of cells, however, expressed NG2 (G-I). All images are from the ventrolateral striatum, Scale bar 10μm (A-C) and 100μm (D-I).

of GFP expressing cells were present in this zone in animals that received the Rv.Isl1 injections. In the region of the striatum furthest away from the LV (Zone III), GFP expressing cells in all section form all animals analyzed in the Rv.Isl1 group could be detected. This is in contrast to the control animals, where no GFP expressing cells were present in this zone in any of the section analyzed. The results from the quantification confirm initial observations that SVZ cells that are manipulated to express Isl1 give rise to a population of cells that ectopically migrates into the striatum.

Summary of paper II

"Retrovirally delivered Islet-1 increases recruitment of Ng2 expressing cells from the postnatal SVZ into the striatum"

We show that ectopic expression of Isl1 in the neonatal SVZ is sufficient to direct the differentiation fate and promote migration of the SVZ cells to the striatum. The cells located striatum express Ng2 and are most likely glia progenitor cells. In the neonatal brain, ectopic Isl1 expression does not interfere with OB neurogenesis. An increased understanding of the plasticity and developmental potential of the SVZ cells, and how to manipulate them, may help in designing new strategies for brain repair utilizing these resident precursor cells.

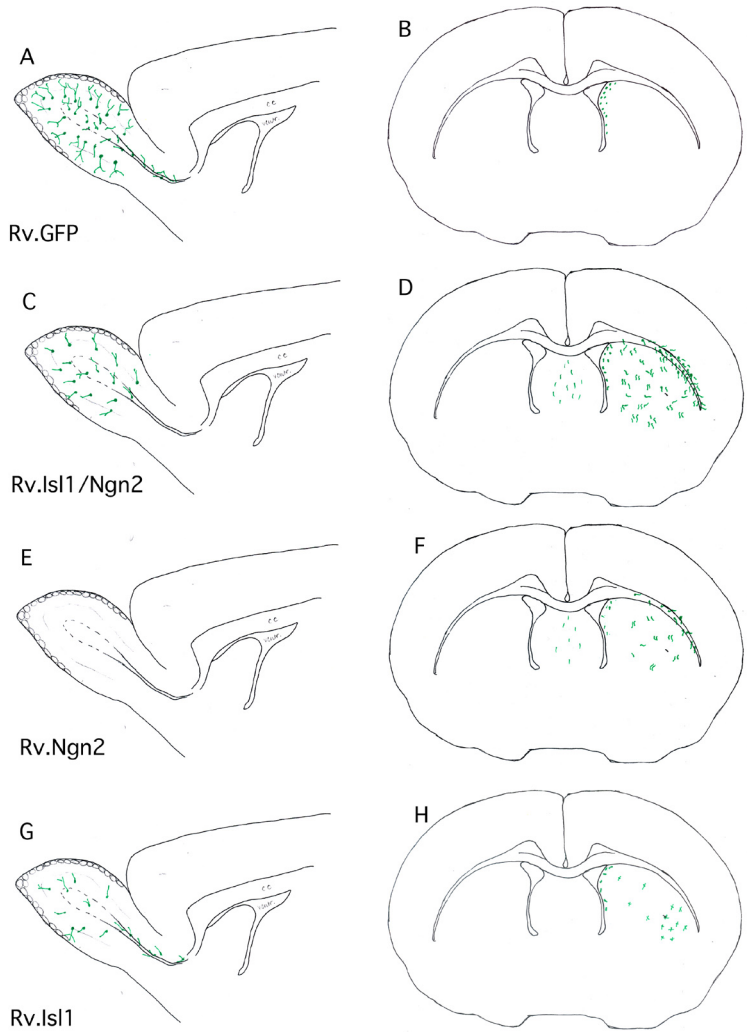

Figure 5.

Schematic pictures of OB, striatum, and CE showing GFP positive cells 2w after viral vectors injections to the lateral ventricle in neonatal rats.

The normal pattern with SVZ cells migrating through the RMS to the OB could be detected by the expression of GFP in animals that received control (Rv.GFP) injections (A and B). This was also the case in the Rv.Ngn2/Rv.Isl1 injected animals (C and D) where transduced cells even were detected in the striatum, CE and septum. After injection with Rv.Ngn2 only (E and F) no transduced cells could be detected in the OB (E). However, transduced cells were present in the striatum and CE (F). In the Rv.Isl1 group (G and H) GFP positive cells could be detected in OB and in the ventrolateral part of striatum but no transduced cells were detected in the CE or the septum.

NEW CELL POPULATION WITH NEUROGENIC POTEINTIAL (paper III)

As described above (in paper II), genetic manipulation with Isl1 of the postnatal SVZ cells contribute to migration into the striatum. To further stimulate the Isl1 transduced cells to differentiate into neurons in striatum we co-transduced the postnatal SVZ cells with Isl1 and the proneural gene Ngn2. The neurogenin transcription factors, are activators of neuronal gene expression (Morrison, 2001) and participate in the specification of neuronal phenotypes during brain development (Fode et al., 2000). In cortical progenitor cell cultures, neurogenin suppresses the formation of astrocytes by inhibiting the expression of glial genes (Sauvageot and Stiles, 2002). We injected in combination retroviral vectors encoding Ngn2 (Rv.Ngn2 with GFP as marker gene) and Rv.Isl1 at the same time to the lateral ventricle of neonates thus infecting the progenitor cells in the SVZ with both Ngn2 and Isl1.

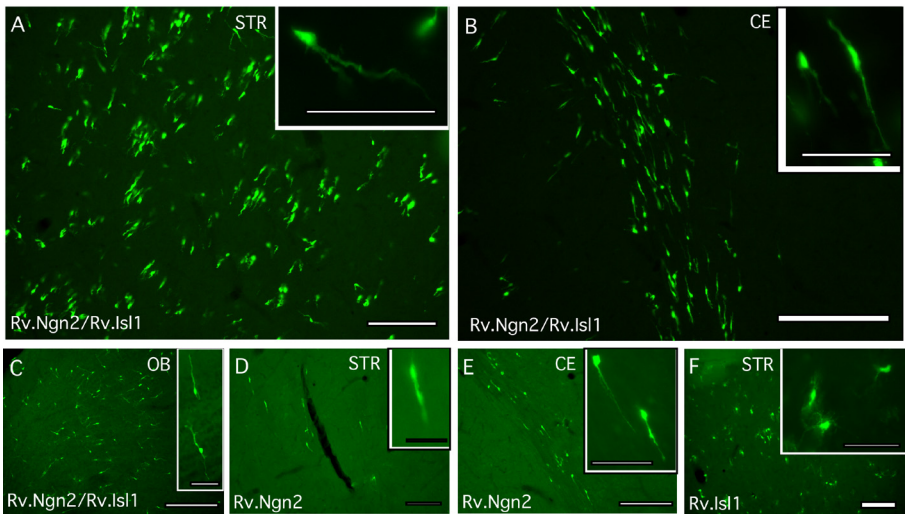

Figure 6.
Neuroblast-like morphology of the transduced cells in striatum and CE.
The cells found in striatum (A) and CE (B) in the Rv.Ngn2/Rv.Isl1 injected group displayed neuroblast-like morphologies. The cells found in OB in this group had the normal morphology of newly derived OB cell (C). In the Rv.Ngn2 injected group (D and E) the cells found in striatum (D) and CE (E) also had a neuroblast-like morphology. The morphology of the transduced cells in striatum after Rv.Isl1 (F) is glial-like. Scale bar 100mm.

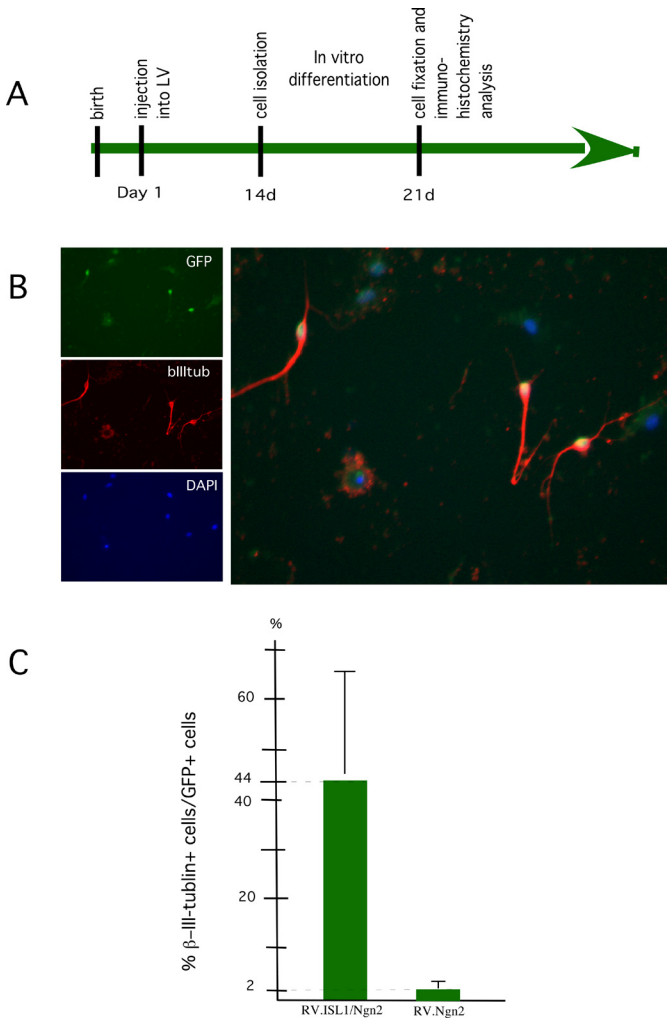

Figure 7.

In vivo Rv.Ngn2/Rv.Isl1 co-transduced cells express b-III-tubulin in vitro.

One day after birth the neonatal rats were injected with Rv.GFP, Rv.Ngn2/Rv.Isl1, Rv.Ngn2, or Rv.Isl1 to the lateral ventricle (A). Two weeks later the transduced GFP expressing cells located in striatum were dissected out and cultured for further differentiation for one week. Immunohistochemical analysis showed co-expression of GFP and b-III-tubulin in cells in the Rv.Ngn2/Rv.Isl1 injected group (B). In this group 44 ±17.5% of all isolated GFP expressing cells (n= 420) expressed b-III-tubulin (C). In the Rv.Ngn2 group, only 2 ±0.85% of the GFP expressing cells (n= 163) were b-III-tubulin positive. Values are expressed as mean ±SEM.

Neuroblast-like morphlogy of the transduced cells in the straitum and CE.

Interestingly, after injection of Rv.Ngn2 in combination with Rv.Isl1 the progeny from the transduced SVZ cells were found to populate the striatum, capsule external (CE), and septum (figure 5). These cells were two weeks post-injection found evenly distributed over the striatum. The cells detected in CE were clustered tight to each other and in the septum and a few scattered cells were found. These newly SVZ derived cells found in the striatum and CE had an elongated cell body and a long process resembling a neuronal-like morphology (figure 6). We could not detect the cells with any cell specific marker for neurons (DCX and MAP2) or glia (Ng2 or APC). So far leaving the phenotype of the transduced cells found in the striatum and CE undetermined. However, the MAP2 analysis revealed, since this neuronal marker does not stain as intense within the white matter that the transduced cells seem to appear in the white matter tract in the striatum. This effect with new cells in striatum after Rv.Ngn2 injection in combination with Rv.Isl1 was most prominent at two weeks post-injections but still after six weeks the transduced cells could be detected. In addition to this, in the normal migration of SVZ progenitor cells to the OB were not disrupted in this co-injected group.

In vivo Rv.Ngn2/Rv.Isl1 co- transduced cells express β-III-tubulin in vitro

To investigate whether the Ngn2 and Isl1 co-transduced SVZ cells recruited to the striatum could differentiate into neurons outside the striatal environment we isolated and cultured the cells. Two weeks after Rv.Ngn2/Rv.Isl1 injections to SVZ the striatum were dissected out and the dissociated cells were grown and further differentiated for one week. Interestingly, almost half of these isolated cells expressed the neuronal marker β-III-tubulin (figure 7). They also showed neuronal morphology in culture. This cell specific marker expression for neurons together with the neuronal morphology indicates that these *in vivo* transduced cells harbor the potential to differentiate into neurons. This effect is only seen with co-transduction of Isl1 and Ngn2.

Summary of paper III

"Expression of Isl1 and Ngn2 in neonatal SVZ results in a striatal cell population with potential for neuronal differentiation".

Ectopic expressions of both Isl1 and Ngn2 to SVZ progenitor cells contribute to novel cells in striatum and CE. These new cells in striatum express neuronal-like morphology but not the neuronal markers we tested (DCX and MAP2). Once their external environment was changed as happened when they were removed from striatum to *in vitro* culture, they expressed the neuronal marker β-III-tubulin. Could change in the brain environment with e.g. lesions or BDNF administration affect the Ngn2 and Isl1 transduced progenitor cells to express neuronal markers *in vivo*. A change in the external environment (e.g. stroke) in combination with intrinsic genetic

change can maybe override the rare event of neurogenesis seen after ischemia and make the neuronal replacement from endogenous stem cells more functionally relevant (Arvidsson et al., 2002).

Concluding remarks and future prospects

CONCLUDING REMARKS AND FUTURE PROSPECTS

The research in this thesis

The important finding this thesis has provided is more knowledge of the postnatal SVZ progenitor cells and their plasticity. From the results presented in this thesis we now know that by addition of developmentally important transcription factors we can influence the postnatal SVZ progenitor cells to a new fate. We have by ectopic expression of Isl1 and Ngn2 to the progenitors cells in SVZ directed their progeny to striatum and CE. With addition of Isl1 alone the newly derived cells in striatum expressed the glia progenitor marker Ng2. Co-injections with both Isl1 and Ngn2 generated a neuronal-like morphology of the striatum and CE derived cells. The Isl1and Ngn2 co-transduced cells, with neuronal-like morphology in striatum, expressed β-III-tubulin *in vitro*. These new observations contributes to our understanding of the SVZ progenitor cells potential but still many questions are unresolved before we fully understand the potential of these cells and cell replacement therapy using endogenous neural stem cells can be reality.

What is solved and unresolved about the postnatal CNS neurogenesis

To day we know that the postnatal brain consists of progenitor cells that generate neurons. Also their location and identity have started to be revealed. Even though many factors have been characterized still a lot is unknown about what mechanisms that regulate the proliferation, migration, differentiation, nerve growth, synaptogenesis, functional integration, survival, and neuronal destiny. We have gained lot knowledge about the neurogenesis since the early 90-ties, but still questions are unresolved such as; how and why does adult neurogenesis persist in only a few brain regions, and what is the function that underlies this residual plasticity in the adult mammalian brain? Maybe it is from studies of the normal brain that these questions will be answered. The documentation and the existence of neural stem cells started the focus of the research in the postnatal normal brain.

Hetrogeneous mixture of cells in the SVZ

An interesting question for future studies to answer is if the newly derived transduced cells that we found in striatum, external capsule and septum have been redirected or directed? It is to day unknown whether the progeny on their way to OB have been redirected to migrate to striatum or if it is direction of a subpopulation of cells that in the normal case proliferate in SVZ but die and don't migrate. Recent reports have suggested that the neonatal SVZ consists of different subpopulations of progenitor cells (Willaime-Morawek et al., 2006b) and that they may have different potentials (Willaime-Morawek et al., 2006a). These suggestions open the possibility that more than one type of multipotent neuronal progenitor cell does exist in the

SVZ. Possibly, one such subpopulation has a special potential to respond to change in the microenvironment or as in this case to Isl1 and Ngn2. In the normal case these subpopulations of cells maybe proliferate to be ready for an extra stimuli response, but when the stimuli does not occur they eventually die. If this is the case then in our study in the control-injected animals we will not detected these cells outside the SVZ/RMS/OB system. However, in the Rv.Isl1 and/or Rv.Ngn2 injected animals the subpopulation of progenitor cells in SVZ now have that extra stimuli to respond to by addition of transcription factors and are now not only ready but also able to migrate and populate new areas.

Good lab technique and methods

The VSV-G pseudotyped retrovirus used in this study label efficiently the dividing cells *in vivo* and works as a potent tool for analyzing migration and fate mapping of proliferating cells. It can also be used in studies where the response of how neural progenitor cells is acting after damages of the brain. Also as in our case the use for transduction of instructive genes to study their effect on the proliferating cells fate. The methods and lab technique is important to provide the right evidence, an example of this is that Altman and Das (1965) because they had good technique could detect the postnatal neurogenesis that others haven't seen. "Will better techniques reveal more neurogenesis in more areas (Gage, 2002)?"

Methods

METHODS

Retroviral vector constructions and production

pCMMP-GFP/neo plasmid was kindly provided by Dr RC Mulligan. The GFP/neo was then replaced by GFP and the woodchuck posttranscriptional regulatory element (WPRE). VSV-G pseudotyped retroviral vectors were produced as described by Ory et al., 1996). A 15 cm petridish confluent to 95% of 293 GPG cells were transfected using lipofectamine (Gibco-BRL). 25 μg of the pCMMP.GFP.WPRE was diluted into 1,3 ml OptiMEM (Gibco-BRL) and incubated at room temperature for 30 minutes with 156 μl lipofectamine diluted in 1,3 ml OptiMEM. The cells were washed twice with Hank´s balanced salt solution containing 1 μg/μl teracycline and incubated for 30 minutes at 37° in 10 ml OptiMEM. The DNA-lipofectamine mixture was diluted with 2,6 ml OptiMEM before it was placed on the cells dropwise. 8 hours posttransfection 10 ml of 293 media (DMEM, 10% fetal bovine serum, 1% glutamine, 25 mM HEPES) was added and less then 24 hours later the media was changed to 25 ml of 293 media. The supernatant was then changed every 24 hours and collected at 48-168 hours. To determine the viral titer, 100.000 293T cells were infected with different volumes of the virus concentrated by ultracentrifugation. The dilution resulting in less than 30% positive GFP cells was used to calculate TU.

Surgical procedures

The pups were anesthetized by hypothermia in water-ice as describe earlier (Nikkhah et al., 1994) and the adult animals with halothane (2% in air). For injections of the vectors to the lateral ventricle and SVZ the animals were placed in a stereotactic frame. 1 μl of the vectors was injected into the lateral ventricle of rat pups (postnatal day 1 or 2). Adult animals received 1 μl into the SVZ or 5 μl to the lateral ventricle. Neonatal animals (2d-1w) the brain was removed from the skull and placed in 4% paraformaldehyde in 0.1M phosphate buffer pH7.4 (PFA) for 24 h. Animals from 2w old to adults were perfused with 4%PFA through the aorta and post-fixed for 4-6h.

Dissection and culturing

The brains were removed from the skull and the region of interests was dissected out. The brain tissue were kept in ice-cold L-15 dissection medium. The tissue piece were incubated at 37°C for 20 min in 0.05% DNase and 0.1% trypsin and subsequently mechanically dissociated into a single cell suspension. The cells were plated in Poly-L-Lysine (PLL)-coated plastic wells (4mg PLL/cm2) at the concentration 200 000 cells/well in DMEM/F12-based supplemented with N2 hormone mix and 1% FBS (Jensen et al., 2004). After 5-7 days of differentiation the cells were fixed in 4% PFA for 15 min at room temperature.

Immunohistology

Immunostainings were done on PFA fixed cells and tissue. The brains were cut in coronal and OB in sagittal sections (30-40mm) on a freezing microtome in series of 5-8 per brain. The sections were kept free flouting in potassium phosphate-buffered saline (KPBS). The cells and tissue were preincubated in blocking solution containing 5% serum and 0.25% tritonX-100 followed by incubation with primary antibodies in blocking solution at room temperature over night. The cells and the tissue were rinsed three times in KPBS and serum. The antibodies were visualised with FITC or Cy3 conjugated secondary antibodies and analyzed using a fluorescence microscope. Information of the primary antibodies used in this thesis is listed on table 1. The sections were then rinsed three times in KPBS, mounted on glass slides and cover slipped with PVA/DABCO. The glass slides were stored in a cold room until analyzed using fluorescence microscope (Leica, Germany) equipped with the appropriate filter boxes. Deconvolution and confocal microscopy was used to detect double labeled cells

Table 1.
Antibodies

Antigen	Supplier	Species	concentration
APC	Calbiochemi	mouse	1:200
β-III-tubulin	Promega	mouse	1:1000
DARPP-32	Chemicon	rabbit	1:1000
DARPP-32	Greengard	mouse	1:20000
DCX	Santa Cruz	goat	1:3000
Er81	Jessel/Morton	rabbit	1:2000
GFAP	DAKO	rabbit	1:500
GFP	Chemicon	chicken	1:5000
Isl1	Edlund	rabbit	1:400
Isl1	Hyb Bank	mouse	1:100
MAP2	Chemicon	mouse	1:200
Nestin	McKay	rabbit	1:500
Ng2	Chemicon	rabbit	1:500
NeuN	Chemicon	mouse	1:100
S100β	sigma	mouse	1:500
TH	Chemicon	rabbit	1:500

Populärvetenskaplig sammanfattning

POPULÄRVETENSKAPLIG SAMMANFATTNING

CELLERNAS ÖDE - SAMTAL OM HJÄRNFORSKNING

Mellan avhandlingens författare Nina Rogelius och skribent Jenny Henningsdotter

- Så du sysslar alltså med hjärnforskning – vad exakt är det du forskar om?
- Jag forskar om de celler som finns i en del av hjärnan som kallas den subventrikulära zonen. En ventrikel är ett hålrum i hjärnan och de celler jag studerat finns i zonen som utgör väggen mellan hjärnan och de laterala ventriklarna.

- Vad är det för speciellt med cellerna inom just detta område?
- Man har länge ansett att nya nervceller inte kan bildas i hjärnan, att den uppsättning man har när man föds har man hela livet. Men nu har man upptäckt att den vuxna hjärnan faktiskt kan bilda nya celler utöver den medfödda uppsättningen! Detta sker dock endast i två av hjärnans områden varav den subventrikulära zonen är en.

- Vilka möjligheter ger den här kunskapen?
- Forskningen inom fältet vill med denna kunskap att hjärnan ska kunna använda de nybildade cellerna i hjärnan till att läka hjärnskador. Läkning med kroppsegna celler är alltid att föredra.

Man vill med andra ord kunna ”fylla på” det skadade området i hjärnan med friska celler och därmed alltså läka hjärnan med hjärnans egna celler. Men det finns ett stort problem. Eftersom nybildningen av celler endast sker i två begränsade områden i hjärnan så kan den inte, vad vi känner till idag, läka skador på samma sätt som exempelvis huden kan. Därför vill forskare kunna styra nya nervceller till skadeområdet för att möjliggöra läkningen.

- Är det möjligt idag att styra cellerna dit de behövs?
- Vad forskare upptäckt är att stamcellernas avkomma har förmåga att migrera iväg från den subventrikulära zonen där de föds. De är då omogna men mognar till nervceller när de nått sitt mål - i det här fallet den olfaktoriska bulben som man hittar längst fram i hjärnan mot näsan till.

Det är en starkt begränsad väg för de celler som fötts i subventrikulära zonen men det ger oss kunskapen om att nya nervceller kan flytta sig och mogna på andra ställen i hjärnan. Det är dessa celler med förmågan att migrera som jag i min forskning har försökt att styra till ett nytt öde.

- Vad är skillnaden på stamcell och hjärncell?
- Först och främst är det så att vad många i dagligt tal kallar hjärnceller i själva verket är flera typer av celler; nervceller - som sköter all signal-ering i hjärnan - och stödceller; astrocyter och oligodendrocyter, vars uppgift är att underlätta nervcellernas förmåga att skicka impulser. Alla hjärnceller har sitt ursprung i en speciell typ av cell; stamcellen. En nervcell är en mogen cell som har ett bestämt öde och en specifik uppgift. Men en nervcell har inte förmågan att dela sig. Stamcellen däremot har förmåga att dela sig och ge upphov till nya celler inom sitt organ.

- Så hur har du gått till väga, vad är det egentligen du har gjort rent praktiskt?
- Jag har modifierat de nyfödda nervcellerna med olika slags gener. Syftet har varit att styra dem till striatum för att de där ska kunna mogna och bli striatum-nervceller.

- Vad är striatum och varför just dit?
- Striatum är ett ytterst intressant område som styr mycket av motoriken av de frivilliga rörelserna. Kunskap om det här området kan underlätta för exempelvis patienter med Huntingtons sjukdom, även kallad danssjuka, där striatums nervceller dör.

- Du nämnde att du modifierat nervcellerna med gener, hur går man till väga för att göra det?
- Man tillför generna med hjälp av virus. Generna måste ända in i kärnan, in i kromosomerna.

Jag har studerat effekten av två stycken gener; Islet1 och Neurogenin2 (Isl1 och Ngn2). De generna har en viktig funktion under den embryonala utvecklingen till att cellen blir just en nervcell.

- Är inte virus till sin natur skadliga?
- De virus vi använder är genmodifierade och kan endast infektera en gång. De har inte förmågan att bilda nya viruspartiklar och kan alltså inte replikera sig utan endast infektera. När ett virus är genmodifierat kallas det virusvektor. En virusvektor är alltså inget riktigt virus utan endast bärare av våra gener som kan fungera som hjälpmedel för att få in generna i nervcellerna.

- Vad ville du åstadkomma med att föra in generna i hjärnan?
- Jag ville åstadkomma en ny migration, ett nytt öde för de celler som föds i subventrikulära zonen och som vanligtvis går vidare till den olfaktoriska bulben. Hypotesen var att de nyfödda cellerna som var bärare av Islet1 och Neurogenin2 istället skulle gå till striatum.

- Och stämde hypotesen?
- Ja! Med de tillförda generna begav sig cellerna till striatum. Vi kunde alltså styra dem dit vi ville och de tog sig an ett nytt öde. Men väl i striatum blev de inte nervceller utan istället omogna gliaceller, ng2 positiva celler. I den vuxna hjärnan bildas ju naturligt inga nervceller i det området och miljön är kanske därför inte gynnande för utvecklingen till nervcell.

Så för att ta reda på om miljön i striatum kan vara avgörande har vi tagit ut cellerna och odlat dem i en skål. Resultaten visar att utanför den hämmande miljön kan de bli nervceller!

- Fantastiskt! Vad kommer denna kunskap att kunna leda till?
- Ökad kunskap om hur påverkningsbara de kroppsegna nyfödda cellerna är, t ex vilka faktorer som de påverkas av eller med vilka faktorer man kan påverka och styra deras öde kan vara av stort värde för alla neurodegenerativa sjukdomar. Vad min forskning visat är att celler kan styras till ett nytt öde. Det kan i sin tur möjliggöra att man i större utsträckning kan använda kroppsegna celler i läkningsprocessen vid skador i hjärnan.

En "aha"-upplevelse är när hjärnan får vetskap
om det som hjärtat och kroppen redan visste

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