The endogenous repair capacity of the parkinsonian brain
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Chapter 6

General discussion
Parkinson's disease (PD) is a neurodegenerative disorder, characterized by the loss of dopaminergic neurons in the substantia nigra. This leads to dopamine deficiency in the striatum, which causes the classical parkinsonian motor symptoms, like tremor and rigidity. In addition, the disease is neuropathologically characterised by α-synuclein-positive cellular inclusions, the Lewy bodies. These inclusions are first seen in the brain stem and may spread throughout the whole cortex. The cortical Lewy bodies may be related to the cognitive changes that are highly prevalent in PD patients (Bosboom et al., 2004).

A major breakthrough in neuroscience is the discovery that the adult brain still contains neural stem cells (NSCs) in the two main neurogenic niches, the subventricular zone (SVZ) (Sanai et al., 2004) and the subgranular zone in the hippocampus (Eriksson et al., 1998). This thesis is focused on the SVZ niche, which produces new neurons for the olfactory bulb. The NSCs of the SVZ are situated close to the striatum, which makes them a good candidate for therapeutic targeting in PD. If this endogenous pool of stem cells could be activated, directed towards the striatum and differentiated into dopaminergic neurons, these cells might help to replenish dopamine levels in the striatum. In this thesis, that contains the first steps on the way to such a therapeutic strategy, we first describe a novel marker for human NSCs in the SVZ of elderly human brain donors, i.e. glial acidic fibrillary protein δ (GFAP-δ) (chapter 2). Subsequently, we show that the pool of GFAP-δ-positive stem cells is spared in PD patients (chapter 3). Then, we demonstrate that the NSCs in the SVZ can form subventricular glial nodules (SGNs) and that factors in cerebrospinal fluid (CSF) may be involved in the formation of these nodules. The nodules are present in neurological controls, PD patients and human immunodeficiency virus (HIV) infected donors. Finally, we study the involvement of cortical astrogliosis in the cognitive decline in PD patients and show that astrogliosis is not prominent in PD but that cognitive decline is related to α-synuclein-positive inclusions.

In this general discussion, we will discuss the variability of the human SVZ architecture and NSCs within and between donors. Furthermore, we will elaborate on our data regarding the presence of NSCs in PD brains, which is in sharp contrast with previously published data that describe a decrease in the proliferation of these cells in the PD SVZ. Finally, we will discuss the potential of endogenous NSCs as a novel therapeutic target in PD patients and we will propose suggestions for further research to find out how these cells can be stimulated to repair the brain.

1. SVZ cytoarchitecture
1.1 GFAP-δ as a new NSC marker

We have shown in chapter 2 that GFAP-δ is an immunohistochemical marker for NSCs. With this method, we observed colocalisation between GFAP-δ and proliferation and NSC markers. In addition, we have set up unique neurosphere cultures from human post mortem SVZ of elderly donors and PD patients. We showed that GFAP-δ expression is also present in these multipotent neurospheres. The key properties of NSCs are self renewal and potential to differentiate into astrocytes, oligodendrocytes and neurons. In chapters 2 and 3, we have shown the multipotency of NSCs in our neurosphere culture system, but self renewal has been difficult to show, because the neurospheres are very difficult to dissociate and subculture. The cells are extremely well attached to each other and harsh enzymatic treatment followed by mechanical dissociation is needed to get a
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single cell culture. This treatment, however, does not result in secondary neurospheres, probably because the NSCs are no longer viable. Other papers have mentioned similar problems with obtaining secondary neurospheres from adult human cultures, both from surgery-obtained cortical material (Arsenijevic et al., 2001) and post mortem-obtained spinal cord material (Dromard et al., 2008). However, all our data support the idea that GFAP-δ-positive cells are indeed NSCs. To ultimately prove that GFAP-δ-positive cells are true NSCs we need additional experiments, because we are culturing the neurospheres from a mixed SVZ cell population and are therefore not entirely sure that they can be formed from GFAP-δ-positive cells. For this purpose, we are currently phenotyping the SVZ cells for the expression of cell surface markers, to be able to specifically isolate GFAP-δ-positive cells with fluorescence-activated cell sorting (FACS). If we can culture the GFAP-δ-positive cell subpopulation and they do form neurospheres, this will give us the possibility to finally prove that GFAP-δ-positive cells are NSCs and can therefore form multipotent neurospheres. If we can specifically isolate NSCs from the human brain using this technique, this will enable us to study these cells in detail with regard to their proliferation and differentiation capacity, which is highly relevant as NSCs are viewed as targets for endogenous repair in neurodegenerative diseases (as discussed in chapter 1). In addition, the neurosphere cultures enable us to develop cell models for different neurological diseases. Our close interaction with the Netherlands Brain Bank enables us to do experiments with these primary human NSCs, which can be an excellent model to test factors that induce precursor cell proliferation and differentiation of specific neuronal populations.

1.2 SVZ anatomy

In chapters 2 and 3, we have shown the expression of different cell type specific markers and cell proliferation markers in the human SVZ in detail, both in aged individuals and in cases with PD. In chapter 2, we show that there is extensive variation in the SVZ width within and between donors, using our newly discovered marker GFAP-δ. This variety was also clear in the expression of proliferating cell nuclear antigen (PCNA), which is expressed in the cell nucleus during DNA synthesis. We quantified PCNA expression in PD and control cases in chapter 3 and found high inter- and intradonor variability. This variety in the human SVZ has not been explicitly described before, but can be found in some figures of previous studies by other groups (Curtis et al., 2005; Marti-Fabregas et al., 2010). It is, however, well known that protein levels in the post mortem human brain can show high intra- and intercase variability (Hynd et al., 2003). The fact that we observe a similar variability in two different markers also suggests that the variability is intrinsic to the human SVZ and not related to the markers we used. It may be interesting, for therapeutic purposes, to explore the intradonor variability. If the rostro-caudal variation is similar in each individual, this may give an indication of which area has the most potential to deliver new neurons.

For human studies, one has to rely on the use of endogenous markers to study cell proliferation, as opposed to animal models, where bromodeoxyuridine (BrdU) can be administered. We have tried to overcome this limitation in our studies by using multiple markers to study precursor proliferation and NSCs in the SVZ. In chapter 2, we show co-localisation of GFAP-δ with the proliferation markers PCNA and Mcm2. In addition,
we could obtain very scarce and precious material from two donors who received a BrdU injection to assess tumour cell proliferation. BrdU labelling showed the same pattern as PCNA expression. In chapter 3, we study the SVZ of PD patients with three different markers, PCNA, pHH3 and GFAP-δ, each showing the same pattern. We have also shown in chapter 2 that PCNA and GFAP-δ expression largely overlap. For PCNA and pHH3, we have performed a double staining in one experiment and did not observe any overlap in staining, in contrast to mice. This might imply that the human SVZ holds two compartments of proliferating cells, one PCNA-positive and one pHH3-positive population.

An alternative explanation is that either PCNA or pHH3 is not a specific proliferation marker in the human SVZ. We also find PCNA expression in ependymal cells, implying that PCNA expression is not limited to precursor cells, which has been suggested before (Funato et al., 1996). PCNA expression in ependymal cells is unexpected, as they are assumed to be post-mitotic (Spassky et al., 2005), although this view has been contended, and ependymal cells may actually function as NSCs (reviewed in (Chojnacki et al., 2009)).

A striking feature of the human SVZ was the occurrence of subventricular glial nodules (SGNs). On first inspection, these structures were reminiscent of small tumours, which was not unthinkable, as the SVZ is thought to be a source of cancer stem cells (Quinones-Hinojosa and Chaichana, 2007). We did, however, not find any evidence that they actually were small tumours, because they did not express the classical tumour marker Ki67. What we did observe was that the SGNs were common in control and PD brains and consisted of neural stem and precursor cells. From our data, we hypothesize that SGNs arise in early life and are formed after local damage to the ependymal layer, possibly by a viral infection, and are a response to exposure of the SVZ to CSF. From our observations in chapter 4, we regard them as a normal, non-pathological phenomenon in the adult brain. It is likely that they are just a local thickening of the SVZ, which functions normally after repairing the initial damage to the ependymal layer in early life, as discussed in chapter 4. To reliably explain the formation and functional consequence of SGNs, animal models should be used to follow these structures through time and trace the progeny of the NSCs they contain. SGNs have already been described to be present in rodents after growth factor treatment (Cooper and Isacson, 2004; Kuhn et al., 1997; Winner et al., 2008a). These models could be adapted to allow detailed studying of the development of SGNs throughout the lifespan of the animals. In addition, viral infection could be used to determine if these are really the cause of ependymal denudation.

In chapter 2, we show PCNA-positive and GFAP-δ-positive cells in the human rostral migratory stream (RMS). The existence of the human RMS has been heavily debated (Curtis et al., 2007; Sanai et al., 2007). We show a clearly visible path of proliferating cells surrounded by a GFAP-δ-positive glial net, as has been described by Curtis and colleagues (Curtis et al., 2007; Kam et al., 2009). Recent data confirm that the human RMS exists and that it contains single migratory neuroblasts (Wang, 2011). Taking together our data and the mentioned studies, it is becoming clear that the adult human brain contains a rostral migratory path, with few migrating neuroblasts. This is in contrast with younger human brains (Alvarez-Buylla, personal communication), primates (Sawamoto et al., 2011) and rodents (Lois et al., 1996), where there is a stream of migrating neuroblasts.
2. Neural stem cells in Parkinson's disease

2.1 The subventricular zone in PD

As discussed in chapter 1, the general idea about the SVZ in the parkinsonian brain is that precursor cell proliferation is decreased. However, in our study in the SVZ of PD patients, as described in chapter 3, we did not find any evidence that precursor cell proliferation is affected by the disease process. We also investigated precursor proliferation in a chronic mouse model for PD, induced by the toxin chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In these mice, we could show dopaminergic denervation of the striatum, but again no difference in cell proliferation. We also failed to see changes in GFAP-δ-positive cells in the olfactory bulb, despite the olfactory deficits that are present in PD (as discussed in chapter 1). There are a number of factors that can explain the discrepancy between our data and the previously published studies, which did show an effect of PD on the SVZ. We will discuss these below.

Previous studies in animal models of PD have yielded mixed results (as discussed in chapter 1). A number of papers show an increase in SVZ cell proliferation (Aponso et al., 2008; Arias-Carrion et al., 2006; Liu et al., 2006; Peng and Andersen, 2010; Peng et al., 2008), others a decrease (Baker et al., 2004; Freundlieb et al., 2006; Hoglinger et al., 2004; Winner et al., 2006; Winner et al., 2010; Winner et al., 2008b), using BrdU labelling and/or immunostaining for Ki-67 or PCNA. Two previous studies showed that there was no change in precursor proliferation using PCNA staining (Marxreiter et al., 2009; Winner et al., 2004). Thus, our data are in accordance with these last studies.

Another indication that the negative effect on cell proliferation that most studies report may not be mediated by a lack of dopamine, is that proliferation is also decreased in transgenic PD models, expressing human mutant α-synuclein and LRRK2, where there is no dopaminergic denervation (Winner et al., 2010; Winner et al., 2008b). This might lead to the conclusion that any effect that induction of PD in animals has on the SVZ, is caused by other experimental factors, such as acute neuronal damage caused by the PD-inducing toxins. Neurogenesis is affected by many physiological and pathological factors and processes (reviewed in Zhao, 2008 #106), and it is conceivable that the neuronal damage induced by the used toxins affects the results in the involved studies (reviewed in (Kim and Szele, 2008)).

Our data in the human SVZ are inconsistent with three previous studies (Hoglinger et al., 2004; O’Keeffe et al., 2009; O’Sullivan et al., 2010). The first of these is most similar to ours in experimental set up (Hoglinger et al., 2004), as these authors have investigated PCNA expression in the SVZ. Therefore, it is surprising that we do not find a similar decrease in PCNA expression. The study by Hoglinger et al., however, has some deficits. Firstly, PCNA expression was quantified in only 4 PD and 4 control cases, which is too few, considering the large variation we see in PCNA expression in the SVZ. Furthermore, these authors have performed parametric statistical tests, while it is highly unlikely that the data were normally distributed. The patients used in this study are also not well described with respect to their clinical characteristics, except that they were treated with L-dopa. Lastly, nothing is mentioned in the materials and methods about the quantification method. Perhaps images were not randomly taken, and results may have been influenced by observer bias. The second study investigated EGFR-positive cells in the human SVZ (O’Keeffe et al., 2009), which are presumed to represent neural precursor cells. However, there is currently no evidence that EGFR expression is limited
to precursors, and it is known that EGF and EGFR levels are decreased in other areas in PD (Iwakura et al., 2005). We have attempted to reproduce the EGFR expression study in the human SVZ, but we did not see the staining pattern that was described in this study (O’Keeffe et al., 2009) and abandoned the experiment when we could not obtain the same antibody that was used in the O’Keeffe study. The most recent study in the human brain describes a negative effect of disease duration and a positive effect of L-dopa use on the number of NSCs in the SVZ (O’Sullivan et al., 2010). The deficiency of this study is the fact that these authors study Musashi1 expression and call this NSC activity, while Musashi1 is actually a marker for both neural stem and progenitor cells, as well as a population of mature astrocytes (Sakakibara and Okano, 1997). This makes it difficult to draw a definitive conclusion about the effect of the PD disease progress and L-dopa therapy on NSCs. To be absolutely sure about the effects that the PD disease process and its treatment have on the human SVZ, more and larger studies should be performed in different, well described patient populations.

Taking all available data from rodent and human studies together, we conclude that dopamine is not an important regulator of SVZ proliferation and that PD patients have a viable population of NSCs.

2.2 Reactive astrocytes in PD

In chapter 5, we have investigated reactive astrocytes in the frontal cortex of patients with PD and with the Lewy body-related dementias, PD with dementia (PDD) and dementia with Lewy bodies (DLB). Reactive astrocytes have the potential to cause neuronal dysfunction (reviewed in (Sofroniew, 2009)), because it is becoming clear that astrocytes are important regulators of synaptic connectivity (Eroglu and Barres, 2010). In fact, in Alzheimer’s disease, cognitive decline correlates with astrogliosis (Fukuyama et al., 2001; Ingelsson et al., 2004). In addition, astrocytes have recently been identified as a potential source of NSCs (Buffo et al., 2008), as discussed in chapter 1. Astrocytes are considered to play an active role in the initiation of PD (recently reviewed in (Halliday and Stevens, 2011)) for a number of reasons. α-synuclein is expressed in normal human astrocytes in culture (Tanji et al., 2001) and in the normal human brain (Mori et al., 2002). In PD, inclusions of α-synuclein have been found in astrocytes (Braak et al., 2007; Hishikawa et al., 2001; Wakabayashi et al., 2000). The distribution of glial cells with inclusions in PD is similar to that of catecholaminergic neurons in the midbrain (Hishikawa et al., 2001) and to that of intraneuronal LBs (Braak et al., 2007). In addition, the amount of astrocytic inclusions correlates with nigrostriatal neuronal loss (Wakabayashi et al., 2000). It also seems that α-synuclein produced by astrocytes is toxic to neurons (Gu et al., 2010). Thus, it is very interesting to investigate astrogliosis in the parkinsonian brain, to determine the exact role they may have in the disease.

We are the first to investigate astrogliosis in the parkinsonian cortex and did not find evidence that there was astrogliosis in the parkinsonian cortex, despite the presence of Lewy bodies, which were especially prevalent in PDD and DLB. Literature on astrocytes in the human substantia nigra and striatum is mixed, but it is clear that there is an astrocytic activation in PD at least in the substantia nigra (Damier et al., 1993; Forno et al., 1992; Knott et al., 1999; Miklossy et al., 2006). If reactive astrocytes in this area can be stimulated to make new neurons in vitro, this might be an interesting target for therapy.
in PD. In fact, one study shows that resident astrocytes in the parkinsonian rat striatum de-differentiate into radial glia-like cells (Wachter et al., 2010). However, the discovery of reactive astrocytes as NSCs is very recent, and much work is needed to determine if the potential of these cells is the same in the human brain, specifically in the parkinsonian brain, and whether they can be stimulated to form new dopaminergic neurons in the substantia nigra (as discussed in chapter 1). This potential could be examined by culturing astrocytes from the parkinsonian substantia nigra and investigating their differentiation pattern.

3. Neural stem cells as therapeutic target in PD

3.1 Substantia nigra

As summarized in chapter 1, there is currently no evidence that precursor cells in the substantia nigra have the capability to replace the degenerated dopaminergic neurons. More studies in animal models are required before this can be considered a realistic therapeutic approach in PD. Moreover, evidence is needed that the substantia nigra also contains multipotent precursor cells in the human brain. This requires extensive immunohistochemical studies and preferably also the culturing of neurospheres from the human substantia nigra.

3.2 Striatum

An important finding from our studies is that the parkinsonian brain still contains proliferating NSCs, apparently in the same numbers as control brains. This suggests the possibility that certain patients might respond to endogenous NSC therapy. The therapeutic value of endogenous SVZ NSCs in PD can be significant, but this will require extensive research. Most likely, a combinatorial approach will be needed, in which proliferation, migration and differentiation are stimulated. So far, realisation of the first two seems plausible, as animal studies have shown good results (as discussed in Chapter 1, paragraph 3.3). In short, proliferation in the parkinsonian SVZ has successfully been stimulated using the dopamine receptor agonist pramipexole (Winner et al., 2009). Precursor proliferation with neuroblast migration into the striatum has been achieved by administration of TGF-α (Cooper and Isacson, 2004; de Chevigny et al., 2008; Fallon et al., 2000), a combination of EGF and FGF-2 (Winner et al., 2008a), and FGF-2 only (Peng et al., 2008). Neuronal differentiation has been shown after stimulation with PDGF (Mohapel et al., 2005), BDNF (Mohapel et al., 2005), and LGF (Gonzalo-Gobernado et al., 2009). Differentiation of SVZ cells into dopaminergic neurons in the striatum is still very difficult to achieve and, so far, has only been shown in two studies. One of the TGF-α stimulation studies shows limited dopaminergic differentiation (Fallon et al., 2000), and grafting of rat dopaminergic chromaffin cells combined with transcranial magnetic field stimulation results in increased dopaminergic neurons in the SVZ and striatum (Arias-Carrion et al., 2004). For therapeutic purposes, it is not logical to transplant exogenous cells in order to stimulate endogenous neurogenesis, since transplantation with exogenous stem cells is already common practice and gives relatively good experimental results. Therefore, most efforts must be put into finding factors that induce dopaminergic differentiation in the striatum. Much is known about the factors that control dopaminergic differentiation in the developing midbrain (reviewed in (Smidt and Burbach, 2007)). These include the factors sonic hedgehog and FGF-8 and the transcription factors PITX3 and...
NURR1. An attempt to induce differentiation of adult mouse NSCs *in vitro* using sonic hedgehog and FGF-8 did result in TH-positive neurons, although they were not of the midbrain phenotype (Papanikolaou et al., 2008). This approach has not been tried *in vivo* yet, but might be promising. Delivery of the factors that will induce dopaminergic differentiation is also a field that needs to be explored. Currently, viral vectors, especially adeno-associated virus (AAV), are considered to be a safe and efficient way to deliver proteins to the brain, and there are many clinical trials applying this technique, also in PD (Bjorklund and Kordower, 2010). These viruses could be injected into the lateral ventricle or directly into the parenchyma close to the SVZ (Mamber et al., 2010). If the timing of the addition of stimulatory factors is very essential, recombinant proteins could be used.

If inducing *in vivo* differentiation in the striatum turns out to be impossible, an alternative approach might be to isolate autologous precursor cells from the SVZ and differentiate these *in vitro* before transplanting them back. It is possible to endoscopically harvest NSCs from the human SVZ and propagate these cells in neurosphere cultures (Westerlund et al., 2005). This method would probably give similar results to transplantation of foetal cells, but without the ethical issues that are always connected to obtaining foetal cells. Another, more promising, alternative source of new neurons might be induced pluripotent stem (iPS) cells (Stadtfeld and Hochedlinger, 2010). The fibroblasts can easily be derived from patients themselves and turned into iPS cells (Takahashi et al., 2007; Yu et al., 2007). These can be differentiated into the needed neuronal cells and can therefore be transplanted in an autologous fashion. iPS cells have been derived from PD patients (Park et al., 2008; Soldner et al., 2009). Furthermore, iPS cells can differentiate into dopaminergic neurons and these cells can survive after transplantation in the striatum of a rat PD model and improve parkinsonian behaviour (Hargus et al., 2010). However, more research into safety issues is needed before this technique can be transferred to human cases (discussed in (Meyer et al., 2010) and (Arenas, 2010)). With the current pace of technical developments in both the fields of endogenous NSCs and iPS cells, it is difficult to predict which technique is most promising for therapeutic purposes. Therefore, we advise to explore both options in parallel with regard to potential, efficacy and safety to identify which approach will give the best benefits for PD patients. Our work can contribute to the field of endogenous NSC research via the isolation and culture of post mortem GFAP-δ-positive NSCs. These cultures can be used to translate protocols that stimulate NSC proliferation and dopaminergic differentiation from animal research into human NSCs, which is an essential step towards an effective therapy for PD patients.

**Final remarks**

In conclusion, the main finding of this thesis is that the SVZ of aged donors still contains multipotent NSCs and that PD patients have a similar number of NSCs. This means that these cells can potentially be stimulated and thus used for repair in the aging and parkinsonian brain. This is an exciting option, but there are many practical hurdles to overcome before patients will benefit from this knowledge. More knowledge is needed about the human SVZ, and its proliferation and differentiation capacity. The isolation of GFAP-δ-positive NSCs may be very useful in this process.