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Neurorestorative strategies involving neurogenesis, neuronal precursors and stem cells in animal models of Parkinson's Disease

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On the Cover:
On the cover is the double labeling of newborn dopaminergic neuron in the adult mice of substantia nigra pars compacta, this newborn neuron shows tyrosine hydroxylase+ (in red) with BrdU+ nucleus (in green).

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To my beloved family for all their love and support

献給我的母亲

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ABSTRACT

The general aims of the thesis is to provide evidence for neurogenesis in the adult substantia nigra pars compacta (SNpc), where the dopamine-producing neurons lost in Parkinson's disease (PD) reside; to optimize methods used for detecting newborn nerve cells in adult brain regions with a low rate of neurogenesis; to explore the mechanism of nerve cell death in animal models of parkinsonism and early degenerative and restorative changes in nigral cell populations; and to develop a therapeutic approach that could translate into a future restorative disease-modifying strategy in PD.

Paper I. We provide evidence for the generation of dopaminergic projection neurons in adult SNpc, and estimate the low rate of turnover in this brain region. We administered the thymidine-analogue 5-bromodeoxyuridine (BrdU) or [^{3}H]-thymidine using various regimen strategies to establish the generation of new neurons in SNpc in the confocal light and electron microscope. To trace the origin of the newly generated cells from stem cells lining the cerebroventricular system, we labeled ependymal cells with 1,1´-dioctadecyl-6,6´-di-(4-sulfophenyl)-3,3,3´,3´-tetramethylindocarbocyanine (DiI) or rhodamine-conjugated latex beads. The results obtained in this work show that (1) the size of the nigral nerve cell population remains constant during a large part of the mouse life span; (2) neurogenesis occurs in the substantia nigra and the BrdU-labeled neurons do not represent DNA repair; (3) the newly generated neurons project to the striatum and integrate into synaptic circuits; (4) there is increased neurogenesis after a partial 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesion.

Paper II. Methodological differences may explain discrepancies between research reports on adult mammalian neurogenesis in the brain outside the widely accepted neurogenic regions, i.e. hippocampus and olfactory bulb/subventricular zone. We describe a method to dissolve and administer BrdU at high concentrations (150 mg/mL) into the adult mouse right cerebral ventricle to demonstrate neuronal incorporation of this thymidine analogue in CNS regions with a low rate of neurogenesis. The dosage regimen, duration and survival time of the mouse after the end of the nucleotide analogue administration all are critical, since e.g. exposure to low doses (paper **I**) did not result in a robust neuronal label. Techniques to optimize BrdU detection include tissue denaturation to fully expose the incorporated nuclear thymidine analogue for immunohistochemical staining. On the other hand, strategies to protect other tissue antigens against deterioration by this denaturation (HCl and pepsin) are necessary. Nigral neuronal incorporation of another proliferative marker, [^{3}H]-thymidine confirmed the presence of neurogenesis in adult mammalian substantia nigra under physiological conditions.

Paper III. To understand early neurodegenerative and neurorestorative post-lesion events in SNpc after a single dose of MPTP, we analyzed cell death by apoptosis as well as parallel dynamic changes in nigral populations of neurons, glia and progenitor cells. At the time of the peak of the MPTP-induced apoptosis, condensed neuronal nuclei containing fragmented DNA and activated caspases in the cytoplasm was found in nigral neurons, but neither in glial nor progenitor cells. Ultrastructural analysis confirmed the neuronal phenotype of cells with apoptotic morphologies. Moreover, the dynamic changes during the first 7 days after a single MPTP-induced lesion indicate that neuronal apoptosis is slow, lasting over several days, and that parallel changes in neuronal and glial progenitor populations occur, possibly representing both neurodegenerative and neurorestorative events. Stereological cell counts of nigral neurons using antibodies against tyrosine hydroxylase and neuronal-specific nuclear protein combined with Nissl staining all provide evidence that neurons are lost by a single dose of MPTP, in contrast to earlier reports indicating that neurons do not die in this lesion model, but merely lose expression of the rate limiting enzyme for dopamine synthesis.

Paper IV. Intracerebroventricular administration of platelet-derived growth factor (PDGF)-BB for two weeks in parkinsonian animal models result in long-lasting dopaminergic restoration and functional recovery. We used several animal models, all inducing partial lesions in the nigrostriatal dopamine system, including mice and monkeys lesioned with MPTP and rats with a 6-hydroxydopamine (6OHDA)-injection in the right medial forebrain bundle. PDGF-BB promoted proliferation of neural progenitor cells in the subventricular zone in all animal species used. When the proliferation inhibitor cytosine-D-arabinofuranoside was coadministered with PDGF-BB in the 6-OHDA lesioned rats for two weeks, both structural and behavioral restoration was blocked. A link between the anti-parkinsonian effect of PDGF-BB and proliferation points to a new strategy in the search for disease-modifying agents that could lead to the development of new therapies against PD.

Keywords: **Parkinson's disease; substantia nigra pars compacta; adult neurogenesis; stereology; apoptosis; dopamine; tyrosine hydroxylase**; **nestin; 5-bromodeoxyuridine; [^{3}H]-thymidine; MPTP, 6-OHDA;platelet-derived growth factor-BB.**

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* These authors contributed equally to the study

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
Ara-C	cytosine-D-arabinofuranoside
BrdU	5-bromo-2´-deoxyuridine
CE	coefficient error
CNS	central nervous system
CTB	choleratoxin subunit B
CV	cresyl violet
DAB	3.3'-Diaminobenzidine
DAPI	4´,6-diamidino-2-phenylindole
DAT	dopamine transporter
DG	dentate gyrus
DESO	dimethyl sulfoxide DiI 1,1´-dioctadecyl-6,6´-di-(4-sulfophenyl)-3,3,3´,3´-tetramethylindocarbocyanine
Dcx	doublecortin
eGFP PRV	enhanced green fluorescent protein expressing pseudo rabies virus,
FG	Fluoro-Gold
icv	intracerebroventricular
IF	interfilament
im	intramuscular
ip	intraperitoneal
ir	immunoreactivity
iv	intravenous
GFAP	glial fibrillary acidic protein
LB	lewy body
MPP+	1-methyl-4-phenyl-pyridinium ion
MPTP	1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine
NeuN	neuronal nuclei
NG2	chondroitin sulfateproteoglycan 4
NSCs	neural stem cells
PBS	phosphate buffered saline
PDGF-BB	platelet derived growth factors-BB
PSA-NCAM	polysialic acid-cell adhesion molecule
sc	subcutaneous
SNpc	substantia nigra pars compacta
SPECT	single-photon electron computed tomography
SVZ	subventricular zone
TdT	terminal deoxynucleotidyl transferase
TH	tyrosine hydroxylase
ßIII-tubulin	Tuj-1
TUNEL	terminal deoxynucleotidyl transferase-mediated Dutp-biotin nick end labeling
PD	Parkinson's disease

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1 INTRODUCTION

1.1 Historical dogma

It has long been thought that neuronal production ceases after birth and that regeneration of the nervous system is impossible. Although suggestions regarding the existence of dividing cells in the postnatal central nervous system (CNS) were raised (Allen 1912, Hamilton 1901, Kriss et al. 1963), it was impossible, using methods of the time, to trace the fate of those rarely dividing cells and to prove that the newborn cells were in fact neurons rather than glia (Ramón y Cajal 1928). Since then, “no new neurons after birth” became a central dogma in neuroscience for almost a century (Gross 2000).

1.2 Neurogenic versus non-neurogenic regions in the adult brain

1.2.1 Neurogenesis in neurogenic regions

The first challenge to the "no new neurons" dogma came in the late 1950s, when Joseph Altman used techniques sensitive enough to detect the ongoing cell division that occurs in the adult brain. Thus,a new method was developed to label dividing cells with [^{3}H]-thymidine, which is incorporated into the replicating DNA during the S-phase of the cell cycle and can be detected with autoradiography (Sidman et al. 1959). The generation of new neurons was first reported using this technique in three-day old mouse brains (Smart 1961). Soon after, Altman and colleagues published a series of papers reporting evidence based on [^{3}H]-thymidine for new neurons in various regions

of adult rats, including the dentate gyrus of the hippocampus (Altman and Chorover 1963), neocortex (Altman and Chorover 1963) and olfactory bulb (Altman and Chorover 1963). However, the limitations of the used methods and the absence of neuron-specific immunocytochemical markers at the time made it difficult to distinguish between glial cells and neurons. These limitations led to a widespread lack of acceptance of these results and made research in the field difficult. Thus, this early work was not immediately embraced.

Later, the studies of Nottebohm and collaborators in the early 1980s on the neurological basis of bird song marked a turning point, showing that there is neurogenesis in adult bird brain, the new cells being generated from the lining of the ventricles (Goldman and Nottebohm 1983). And those studies on birds facilitated the 'rediscovery' of hippocampal neurogenesis in adult rodents in the early 1990s (Gould et al. 1998a). Adult neural stem cells, the source of new neurons, were first isolated from the adult central nervous system of rodents (Reynolds and Weiss 1992) and later from humans (Kukekov et al. 1999). Around this time, there were several developments that finally established the presence of neurogenesis in the dentate gyrus of the adult rat. One was using a retrograde fluorescent tracer labelling granule cells of the dentate gyrus through their mossy fiber axons. Another important development, was the induction of thymidine analogue bromo-deoxyuridine (BrdU) as an alternative to tritiated thymidine, a marker of the S-phase of the cell cycle, was introduced to study neurogenesis in adult central nervous system (Corotto et al. 1993, Luskin 1993, Morshead and van der Kooy 1992, Seki and Arai 1993). In addition, important advance was the use of cell-type specific markers for the immunohistochemical identification of the newly generated cells. These studies helped to confirm that neurogenesis occurs in the adult mammalian brain, i.e. that new neurons are continuously generated in some regions of the adult CNS. These regions with the highest neurogenic activity in the rodent brain are the olfactory bulb and the dentate gyrus of hippocampus (Cameron et al. 1993, Eriksson et al. 1998, Lois C 1994, Luskin 1993). Neurogenesis has been convincingly demonstrated in all the mammalian species studied, including the adult monkey (Gould et al. 1999) and the human brain (Eriksson et al. 1998).

Adult *neural stem cells* (NSCs) have two characteristics, (i) self-renewal, with the unlimited ability to produce *progeny* which is indistinguishable from themselves, is proliferative and undergoes mitosis; (ii) multipotency, giving rise to multipotent *progenitors,* which are proliferative cells with only limited self-renewal being able to differentiate into at least two different cell lineages (McKay 1997, Reynolds and Weiss 1992). Lineage-specific progenitors are cells with restriction to one-distinct lineages progenitors that are differentiated into neurons, astrocytes and oligodendrocytes in the central nervous system. Both stem cells and progenitors are referred to as precursor cells (Abrous et al. 2005, Emsley et al. 2005).

Adult mammalian neurogenesis was as mentioned above first discovered in 1965, but only recently has it become generally accepted as a general phenomenon that occurs in all mammals studied, from mouse to human. Thus, new neurons are continuously generated in the adult mammalian CNS throughout life in the subventricular zone (SVZ) and hippocampal dentate gyrus (DG), regions referred to as *neurogenic regions*. Moreover, there are significant numbers of multipotent neural precursors in many parts of the adult mammalian brain (Gage 2000, Gross 2000, Lie et al. 2004). This was a turning point for the field of adult neurogenesis, leading not only to acceptance of the phenomenon, but to a great deal of enthusiasm and curiosity about what it could mean for brain function and repair (Kokovay et al. 2008).

1.2.2 Neurogenesis outside the established neurogenic regions

Cumulative evidence has shown that new neurons are generated also in other parts of the brain (Rietze et al. 2000), including the cerebral cortex (Gould et al. 1999, Magavi et al. 2000), substantia nigra pars compacta (Shan et al. 2006, Van Kampen and Robertson 2005, Zhao et al. 2003), amygdala (Bernier et al. 2002, Fowler et al. 2008), brain stem (St-John 1998), striatum (Bedard et al. 2002, Dayer et al. 2005, Luzzati et al. 2006, Luzzati et al. 2007) and hypothalamus (Kokoeva et al. 2005). Due to the low numbers of neurons incorporating BrdU in these regions, it is difficult to detect these newly generated neurons in the above regions because of the added “noise” of the large non-neuronal BrdU-labeled cell population (Gould 2007).

Local microenvironmental influences on the behavior of precursors, and dividing precursors in these regions can display a restricted differentiation pattern depending on their site of residence. Therefore, important conceptual distinction has been suggested to exist between established neurogenic and other non-neurogenic regions.

Neurogenesis has been demonstrated in substantia nigra pars compacta (SNpc) both under physiological and lesion-induced conditions (Zhao et al. 2003). The identification of adult neurogenesis in this region could potentially be extremely important clinically in relation to PD. If it is possible to stimulate endogenous neurogenesis to replace lost cells, one could modify the disease, and restore some the functionality of the degenerating nigrostriatal neuronal pathway in PD.

Lie and colleagues (Lie et al. 2002) characterized a proliferating population of cells using BrdU throughout the entire midbrain including the substantia nigra, but these cells expressed the oligodendroglial progenitor marker NG2. They did not observe BrdU in nigral neurons. The authors concluded that neurogenesis in the adult substantia nigra, if present, must be an extremely low-frequent event.

However, consistent with our findings, a recent study on dopaminergic neurogenesis within the substantia nigra suggests the existence of a non-proliferative precursor population that can give rise to new dopaminergic neurons (Shan et al. 2006). This group devised novel Nestin-LacZ transgenic mice in which the enhancer element for the neural progenitor protein nestin controls the expression of a LacZ reporter gene. In addition they used BrdU to label newborn cells. The other reports confirming adult nigral neurogenesis all used systemic BrdU administration, and reported newly generated nigral neurons under certain conditions, e.g. after a partial injury or induced by pharmacological interventions (Peng et al. 2008, Shan et al. 2006, Van Kampen and Eckman 2006, Van Kampen and Robertson 2005).

1.3 Regulation of neurogenesis

A variety of environmental, behavioural, genetic and neurochemical factors may influence the proliferation and survival of new cells.

1.3.1 Neurotransmitter

Dopamine is one of important neurotransmitters, which is implicated in the regulation of mood, motivation and movement. It is known to be a positive regulator of endogenous neurogenesis. A decreased proliferation of stem/progenitor cells in the SVZ is found following ablation of the dopamine input to the striatum and SVZ in animals, and in the brains of PD patients (Borta and Hoglinger 2007). An early symptom in PD is reduced olfaction, and it can be speculated that this is due to a reduced neurogenesis in the olfactory bulb (Ziemssen and Reichmann 2007). Therefore treatments that promote neurogenesis in the SVZ could conceivably be beneficial against impairments of nigral neurons in PD.

Van Kampen and Robertson (2005) concluded that chronic D3 receptor stimulation with 7-OH-DPAT triggers a strong induction of cell proliferation in the rat substantia nigra promoting the expression of a dopaminergic phenotype in some of these newly generated cells (Van Kampen and Robertson 2005). They demonstrated triple labelling with the dopaminergic marker TH, the neuronal marker neuronal nuclear antigen (NeuN) and BrdU after chronic infusion of the D3 dopamine receptor agonist 7-OH-DPAT into the lateral ventricle of adult rats.

1.3.2 Neurotrophic factor – a heterogenous group

Cell health and survival is promoted by trophic factors, which operate during development and adulthood. There is no strong evidence that PD is caused by a trophic factor deficiency, but neurotrophic factors are considered attractive as possible neurorestorative or neuroprotective therapeutics. Identified neurotrophic factors include Basic fibroblast growth factor (FGF-2), Brain-derived neurotrophic factor (BDNF), Glial cell-derived neurotrophic factor (GDNF) and Platelet-derived growth factor (PDGF). Although several of these factors have been effective in animal models of PD, clinical trials have not been encouraging. It is possible that a combination of trophic factors is needed for neuronal restoration or the effect is likely to be indirect, mediated via other cells than nerve cells. Interestingly, the existence of yet unknown factors is suggested by

the beneficial effects of undifferentiated cell lines on regeneration processes (Richardson et al., 2005)

PDGF appears to play an important role in the normal development of the CNS and in the adult, and is highly expressed in the SVZ during the peak of the embryonic proliferative and migratory stages. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signalling (Jackson et al. 2006). The substance has been suggested to support neuronal differentiation (Johe et al. 1996, Williams et al. 1997) and acts as a mitogen for neural precursor cells (Ishii et al. 2008) and is up-regulated following injury to adult dopaminergic neurons (Funa et al. 1996). Administration of PDGF into the brain has been shown to induce striatal neurogenesis in adult rats with 6-hydroxy dopamine (6-OHDA) lesions (Mohapel et al. 2005).

1.4 Neural adult stem cell response to injury

Loss of neurons is thought to be irreversible in the adult human brain, because the dying neurons are not considered to be replaced in sufficient numbers. This inability to generate replacement cells is thought to be an important cause of neurological disease and impairment (Eriksson et al. 1998).

Adult neurogenesis opens possibilities to repair the adult CNS after injury or degenerative neurological diseases using cell replacement therapy (Lindvall and Bjorklund 2004, Steiner et al. 2006). In other words, endogenous neuronal production in the DG and SVZ is expected to provide a continuous source of new neurons that replace degenerated neurons in the injured brain. Recent studies indicate that adult neurogenesis is modified by various brain insults including stroke, epilepsy and neurodegenerative disorders (Kaneko and Sawamoto 2009).

1.5 Parkinson's disease

Parkinson's disease is a common neurodegenerative disease with the cardinal signs of resting tremor, slow body movement (bradykinesia) and rigidity (Carlsson 1993, Hornykiewicz 1993). Symptoms are largely attributable to a progressive loss of

dopaminergic neurons in the SNpc (Ehringer and Hornykiewicz 1960), and as a consequence an impaired dopaminergic input to the striatum and other brain regions (Bernheimer et al. 1973).

1.6 Animal model of PD

Animal models are an important tool in experimental medical science to better understand pathogenesis of human diseases. The experiments in this thesis are mainly based on animal models of parkinsonism. Here we describe two major toxin models of PD, induced by exposure of animals to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-OHDA, respectively.

1.6.1 The MPTP model

The development of this model is based on an accidental discovery made in the early 1980s (Davis et al. 1979, Langston and Ballard 1983). MPTP causes systemic mitochondrial complex I inhibition as seen in also many patients with PD (Schapira et al. 1990). The toxic mechanism of MPTP is shown in Fig. 1.

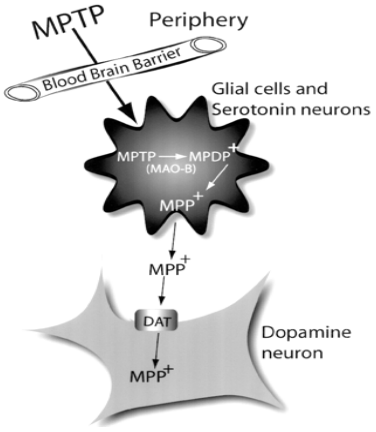

Fig. 1. The toxic mechanism of MPTP

MPTP is highly lipophilic and readily crosses the blood–brain barrier. It is converted by monamine oxidase B to its toxic fully oxidized metabolite MPP^+, which is then actively taken up by dopaminergic neurons via the high-affinity dopamine transporter (DAT). MPP^+ is actively accumulated into mitochondria, where it inhibits complex I of the mitochondrial respiratory chain.

MPTP administration was subsequently shown to model parkinsonism in both mice and primates, and the models have several characteristics in common with the human

neurodegenerative disorder, PD, including degeneration of nigrostriatal neurons and characteristic changes in behavior (MPTP causes tremor, rigidity, akinesia and postural instability in the non-human primate). However, the model is not identical to PD, as there is for example a lack of Lewy body (LB) pathology (Forno et al. 1986, Langston et al. 1999). So far, the MPTP model represents the best-characterized animal parkinsonian model and fulfils many of the criteria for relevant model of the disease.

1.6.2 The 6-OHDA model

6-OHDA was the first chemical agent to be discovered by Porter et al., (Porter et al. 1963) who showed that 6-OHDA induces efficient and long lasting noradrenaline depletion in sympathetic nerves to the heart. It represents one of the most common neurotoxins used in degeneration models of central catecholaminergic projections, including the nigrostriatal system, *in vivo* and *in vitro* (Blum et al. 2001, Sachs and Jonsson 1975, Ungerstedt 1968, Ungerstedt 1976). The mechanism of toxicity is considered to involve generation of free radicals and it is an effective toxin in rats, mice, cats and primates. It has been predominantly used to produce unilateral lesions. In rats, the extent of dopamine depletion can then be assessed by examining rotatory behavior in response to amphetamine or apomorphine (Olds et al. 2006, Ungerstedt 1976). It has therefore proved to be useful for pharmacological screening of agents acting via dopamine and its receptors.

The effects of the neurotoxins 6-OHDA and MPTP in a variety of species, and their advantages and disadvantages have been discussed extensively, including their relevance for mechanisms and pathological properties involved in PD pathogenesis (summarized in Table 2.as following, adapted from Schober A, 2004).

Table 1. Key properties of 6-OHDA and MPTP animal models of Parkinson's disease.

Model	Symptoms induced	Pathology	Favorite applications	Disadvantages
6-OHDA	Unilateral: rotation after, e.g. apomorphine treatment, bilateral: akinesia	Loss of ·Striatal DA- levels ·Striatal TH-ir fibers ·Nigral TH-ir neurons	Tests of preclinical therapies, tests of new pharmacological agents and genetic therapeutic strategies	Acute damage of the DAergic system, unilateral effects, intracerebral injection
MPTP	Akinesia, rigidity and tremor (not in rodents)	Loss of ·Striatal DA-levels ·Striatal TH-ir fibers ·Nigral TH-ir neurons · α-Synuclein aggregation (non fibrillar)	Tests for neuroprotective and neuro-restorative treatments	Acute damage of the DAergic system, non-progressive in contrast to PD, rare generation of inclusion bodies

DA dopamine, *DAergic* dopaminergic, *ir* immunoreactive, *TH* tyrosine hydroxylase

1.7 Treatments

Although L-dopa is an excellent therapy in PD, it is not known to have effects that can alter the progressive course of the disease. The currently available pharmacological and non-pharmacological treatmentsa are able to offer substantial symptomatic relief for patients, without stopping the progressive disappearance of dopaminergic neurons in SNpc (Nutt and Wooten 2005). Thus, a significant medical need for treatment strategies that could modify the disease and restore some of the functionality of the degenerating nigrostriatal neuronal pathways replaced in the brains of PD patients has become a primary goal (Ravina 2003). Recently, experimental approaches to slow disease progression in PD have focused on neuroprotective and neurorestorative actions (Bartus 2009).

1.7.1 Neurogenic Pathway

Cell-replacement therapies may provide a more effective alternative. However, the effectiveness of fetal tissue or stem cell transplants has varied, and issues of tissue availability and ethical concerns limit such transplantation procedures (Freed et al. 2001, Lang et al. 2006, Tatton et al. 1998).

The establishment of neuroregenerative strategies that can compensate for the neuronal loss is attractive. The potential helpfulness of such intervention is supported by the recent demonstration of ectopic migration of newly generated neuroblasts and the proliferation of neural stem/progenitor cells in the SVZ, and both taking place under pathological conditions (Kaneko and Sawamoto 2008, Kanekoa 2008). If endogenous neurogenesis could be increased to a sufficient degree, this would represent an attractive therapeutic strategy in PD.

1.8 Apoptosis

Cell death in the nervous system is an important cellular process for normal development. However, following cell stress and brain injury, cell death becomes unregulated and leads to pathological outcomes and neurodegenerative disorders (Ravina 2003). They include PD and Lewy-body associated disorders (Anglade et al. 1997, Bredesen et al. 2006, Mochizuki et al. 1997, Tatton and Kish 1997, Tatton et al. 1998, Tompkins et al. 1997), and several other human neurodegenerative disorders, including Alzheimer's disease (AD) (Lassmann et al. 1995), and Huntington's Disease (Portera-Caillau et al. 1995). However, other reports have challenged the view that neurons die by apoptosis in PD patients (Banati et al. 1998, Dragunow et al. 1995, Kosel et al. 1997). This controversy is difficult to resolve, since the relationship between the number of apoptotic neurons found in a brain region and the resulting neuronal loss over time has not been established in the adult central nervous system. In addition to the characteristic cellular morphology of apoptosis, neuronal expression of its known effectors, including the major downstream executioner activated caspase-3, have been shown in post-mortem midbrain tissue from PD patients ((Anglade et al. 1997, Hartmann et al. 2000), Caspase-9, activated up-stream of caspase-3, has also been implicated playing a role in nerve cell death associated with neurodegenerative disorders, including PD (Hartmann et al. 2000).

The evaluation of these neurodegenerative events in the human brain is further complicated by possible disease-mediated effects on neuronal progenitor cells and adult neural stem cells. If partial neurorestorative processes paralleling neurodegeneration could be explained, because the paradox of surprisingly high numbers of apoptotic neurons in the human brain, and their presence at late stages of the neurodegenerative disorder, when a large proportion of the neuronal population should already have been lost (Novikova et al. 2006). In line with such more complex models of neurodegenerative disorders involving both neuronal death and partial neurorestoration, affected brain regions from post-mortem analysis of Creutzfeld Jacob's and AD show increased levels of the intermediate filament nestin, a marker for progenitor cells (Lu et al. 2005, Mizuno et al. 2006). Opposite reports have implied that a decreased proliferation of stem/progenitor cells in the SVZ is found following ablation of the dopamine input to the striatum and SVZ in animals, and in the brains of PD patients (Borta and Hoglinger 2007).

A link between MPTP toxicity and apoptosis was first demonstrated *in vitro*, showing MPTP-induced apoptosis of dopamine neurons (Mochizuki et al. 1994). Nowadays, apoptosis has been observed in *in vivo* and *in vitro* models of PD based on the toxicity of 1-methyl-4-phenylpyridinium (MPP^+). MPTP administration to mice can produce varying degrees of permanent damage to the nigrostriatal DA system depending on the schedule of MPTP administration and survival period after MPTP (Seniuk 1990). The underlying death mechanism was later demonstrated after administration of MPTP *in vivo* using a low dose (Novikova et al. 2006, Spooren et al. 1998, Tatton and Kish 1997), but not a high dose regimen(Jackson-Lewis et al. 1995).

Several animal models of neurodegeneration have shown a close relationship between targeted apoptosis and adult *in vivo* neurogenesis, e.g. in the dentate gyrus of hippocampal formation (Gould and Tanapat 1997), but also in regions outside the generally established neurogenic regions, e.g. in neocortex (Magavi et al. 2000). In analogy, reports on an increased MPTP-induced nigral neurogenesis have been published (Peng et al. 2008, Shan et al. 2006, Zhao et al. 2003), and the complexity of these processes in the rostral migratory stream (He et al. 2006).

1.9 Methodological consideration related to adult neurogenesis

We interpret studies on enhanced nigral neurogenesis as an argument that methodological differences are likely to be the most decisive explanation for discrepancies between research groups analysing neurogenesis in brain regions with a low rate of neurogenesis under physiological conditions, i.e. outside the dentate gyrus and olfactory bulb/subventricular zone.

To show that a new neuron has been produced in the adult brain, cell division must first be demonstrated. [^{3}H]-tymidine autoradiography has been the traditional method for identifying such cells. This technique requires injecting [^{3}H]-tymidine into live animals and then examining their brains at different survival times after injection. The isotope is incorporated into the cell during DNA synthesis in preparation for mitosis, and labelling is passed on to the progeny, this combined with various techniques can be used to determine the phenotype of the daughter cells. It has been used to study cell proliferation and neurogenesis in the developing and adult CNS (Altman and Chorover 1963, Cameron et al. 1993, Langston et al. 1999, Sidman et al. 1959).

Presently, studies on neurogenesis are mainly based on the thymidine analogue BrdU, which, like [^{3}H]-thymidine, is incorporated in the S-phase cells (Miller and Nowakowski 1988). BrdU then labels proliferating cells and their progeny, but BrdU is detected by immunohistochemical methods and thus allows double- and triple-labelling with cell type-specific markers and confocal microscopic analysis and stereological estimates of the total number of new cells (Kuhn et al. 1996). However, the use of thymidine analogues and particularly BrdU has raised some technical issues. One question is whether BrdU labelling might identify cells undergoing DNA repair rather than proliferation; this possibility has recently been ruled out for standard BrdU labelling, and has been discussed in detail elsewhere (Cooper-Kuhn CM 2002). There is no evidence that adult neurogenesis detected with standard BrdU assessment can be confused cells with ongoing DNA repair (Palmer et al. 2000). There is some evidence that dying neurons can enter an abortive cell cycle that includes an S-phase (Kuan et al. 2004) and thus could potentially incorporate BrdU. However, such terminally sick neurons do not

survive for long periods. Hence experiments with long survivals do not risk confusion. An additionally important approach is to study the development of labelled cells from at least two different times of investigation. If, for example, early after the pathological event, only immature cells or precursors contain BrdU (identified by a combination of immature morphology, migratory location and early-stage markers), and only at long survival times mature neurons are labelled, this eliminates confusion by ruling out BrdU incorporation by dying cells. Pre-labelling of pre-existing neurons by a variety of methods also eliminates the risk of misinterpreting older cells as newborn. In combination, these and other rigorous analyses can unequivocally identify newborn neurons.

A second methodological issue concerns criteria for identification of cellular phenotype. Presently, NeuN has become the standard immunocytochemical marker to identify neurons, and glial fibrillary acidic protein (GFAP) and S100β are used as astroglial markers. Nestin (the best-known precursor cell marker *in vivo*) was found to be specifically expressed in neural progenitor cells (Lendahl et al. 1990). As neurons and glia differentiate, nestin-expression is down-regulated and replaced with more mature filaments, e.g. neurofilament in neurons and GFAP in astroglia (Lendahl et al. 1990, Steinert and Liem 1990). The presence of proliferating nestin-positive cells in substantia nigra with a potential to differentiate into neurons has been described (Lie et al. 2002).

However, the limits of its usefulness are not fully explored. In mice hippocampus, nestin is notoriously difficult to detect, except under pathological conditions, when it appears to be up-regulated. More recently, mouse-Musashi, a neural RNA-binding protein expressed in proliferating neuronal and/ or glia precursor cells has been used (Kaneko et al. 2000). Other markers of great interest, for example SOX-2 (D'Amour and Gage 2003), and GFAP (Doetsch et al. 1999a) have been described in the SVZ. The development of transgenic mice expressing GFP under a neural-specific element of the nestin promoter has been very helpful in identifying precursor cells *in vivo* (Yamamoto et al. 2001).

1.9.1 The mode of BrdU administration

BrdU has been a principal marker for proliferating cells in studies of adult neurogensis (Gratzner 1982), it can be delivered by intracerebroventricular (i.c.v.), intraperitoneal (i.p.), intravenous (i.v.) injection, or orally for studying adult neurogenesis.

BrdU is metabolized rapidly through dehalogenation in the plasma (the half-life of BrdU in human plasma is reported to be around 8–11 min) (Kriss et al. 1963). The concentration of BrdU that reaches the brain is therefore only a fraction of the administered dose. Hence, i.c.v. injection leads to higher concentration of BrdU in the brain than peripheral delivery, and is employed when a higher and local concentration of BrdU in the brain is sought (Altman and Chorover 1963) and (Zhao et al. 2003). However, i.p. or i.v. injections are the most common mode of administration of BrdU in rodent and primate for studying adult neurogenesis, as they obviate the need for surgery (Kuhn et al. 1996). Oral delivery may be preferred for studying adult neurogenesis, as invasive procedure may induce stress, a condition reported to affect the rate of neurogenesis (Gould et al. 1998b). This simple procedure presents the disadvantage that subsequent cell divisions lead to a dilution of the nuclear BrdU content. Morever, toxicity of BrdU at high doses and the general use of non-saturating doses of BrdU can result in inconsistent or partial labelling of proliferating cells in neurogenesis studies. In this respect, concerns are frequently raised on with regard to the possibility that factors and treatments used to manipulate the level of neruogenesis might also influence the bioavailability of BrdU (Couillard-Despres et al. 2005). Labelling is conceptually similar to labelling with [^{3}H]-thymidine analogue; BrdU is injected into adult animals, where it is picked up by cells synthesizing DNA in preparation for division. These progenitor cells for the remainder of their life, unless the cells undergo consecutive proliferation, which will exponentially dilute the BrdU signal in the nucleus (Ward et al. 1991). Although widely used to detect *in vivo* neurogenesis, labeling with BrdU is problematic.

It has been reported that BrdU is apparently transported from the bloodstream into the brain through the cerebrospinal fluid via an active transport system in the choroid plexus, and only minimally through the blood-brain-barrier (Spector and Berlinger 1982). When there are no labelled cells in a section processed for BrdU immunohistochmistry, it can also be difficult to tell whether this reflects a true absence of S-phase cells or a problem with the processing, e.g. that a step of the processing was left our on that section. It is

always a good idea to process several sections together on the same slide or in the same well, so that identically processed section can be compared. BrdU labelled cells should be stained throughout the nucleus. There is a potential problem, depending on the application mode (duration, concentration of applied BrdU, survival times after BrdU injection), the number of labeled cells within the brain can vary (Cameron and McKay 2001, Prickaerts et al. 2004). Additionally, treatments that disrupt the blood-brain-barrier, eg. kainate or seizures (Oztas et al. 1991, Pont et al. 1995), or alter the flow blood to the brain, e.g. exericise (Ide and Secher 2000), might therefore induce increases in the number of BrdU-labelled cell. This increase, however, might be independent of changes in proliferation, since the effects are attributable to altered BrdU availability in the brain. It should be noted that 300mg/kg is high enough to require labelling all S-phase cells in the adult DG, but it is not necessarily sufficient for studies of other brain regions (Cameron and McKay 2001).

Finally a number of endogenous proliferative markers, also used in earlier studies, include Ki-67, a nuclear protein expressed in dividing cells for the entire duation of their mitotic activity (Endl et al. 2001, Kee et al. 2002). The proliferating cell nuclear antigen (PCNA) allows assessment of cell proliferation as well. Phosporylated Histone H3 (p-H3) expression, which is confined to cells in the G_2 and M phases of the cell cycle (Hendzel et al., 1997) and R1 (Zhu et al. 2005).

2 AIM OF THE STUDY

Ultimately, the aim of basic research must be to serve human beings, especially those who suffer from disease.

The goal of this thesis work was to contribute to the finding of new cures for treatment of PD.

I. **Neurogenesis in the adult mammalian mouse brain**
   To provide evidence that neurons can be generated from endogenous stem cells/progenitor cells both under physiological condition and the injured brain in particular, in the substantia nigra pars compacta, the region where the dopamine-producing neurons lost in PD reside.
II. **BrdU infused into the cerebral ventricle of adult mice labels nigral neurons under physiological conditions - a method to detect newborn nerve cells in regions with a low rate of neurogenesis**
   To explain the discrepancies reported on adult mammalian neurogenesis in the brain outside the widely accepted neurogenic regions we optimized the method to detect newborn nerve cells with a low rate of neurogenesis
III. **Neuronal cell death mechanism under caspase activation involved with an increase in neuronal nestin expression in MPTP mice**.
   To analyze cell death by apoptosis involving activated caspases and the complex early degenerative and restorative changes in nigral populations including neurons, glia and progenitor cells in a PD animal model.
IV. **PDGF-BB infused into the cerebral ventricle provides a restorative therapy in animal models of PD**
   To investigate functional and structural signs of a restorative therapy in different PD animal models via pharmacological activation of endogenous progenitor cells and proliferation of neural progenitors in the adult brain.

3 MATERIALS AND METHODS

3.1 Experimental Animals

Several animal models of parkinsonism were used in the experiments, including monkeys, rats and mice. The animals had access to food and water *ad libitum* and were housed in temperature- and humidity-controlled rooms. All experiments were approved by the local ethical committee on animal experiments and carried out in accordance with the European Communities Council Directive 86/609/EEC on care of laboratory animals.

C57 Bl/6 mice (Papers ***I***, ***II***, ***III*** and ***IV***)

Adult male C57 Bl/6 mice (2–20 months old, B&K Universal or Taconic) were kept 3-6 per cage on a 12h light/dark cycle and had free access to toys including vertical stands for climbing and tunnels.

Sprague-Dawley rats (Paper ***IV***)

Male Sprague-Dawley rats weighing 280-350 g were kept individually on a 12 h light/dark cycle.

Cynomolgus monkeys (Paper ***IV***)

Sixteen drug-naïve female cynomolgus monkeys (Macaca fascicularis) were housed in individual primate cages (1.1 m × 0.8 m × 1 m) under controlled conditions of humidity (50 ± 5%), temperature (24 ± 1°C) and light (13 h light/11 h dark cycles, time lights on 8:00 am). Veterinarians skilled in the healthcare and maintenance of non-human primates and rodents supervised all animal care.

3.2 Surgery

3.2.1 Anaesthetics and infection prophylaxis

Mice were deeply anaesthetized with chloral hydrate or pentobarbital (both 60 mg/kg, i.p.).

Sprague-Dawley rats were anaesthetized with Halothane inhalation.

Female monkeys were pre-treated with atropine-SO_4 (0.04 mg/kg, i.m.) prior to preparation for surgery. At least 10 min later, the animals were anaesthetized with ketamine HCl (10 mg/kg, i.m.). After administration of xylocaine spray, the animals were intubated and maintained in anaesthesia inhaling isoflurane via a volume-regulated respirator. Temgesic or buprenorphine (0.02 mg/kg, i.m.) was administered before and after surgery. For infection prophylaxis, the animals were given Cefotaximer (50 mg/kg, i.v.) and Clamoxyl (30 mg/kg, s.c.) prior to and after surgery. Before brain imaging with Single Photon Emission

Computerized Tomography (SPECT), animals received ketamine (15 mg/kg, i.m., Imalgene, Centavet).

3.3 Administration of substances in vivo

3.3.1 Neurotoxin

Partial lesions of the nigrostriatal dopamine system were obtained with the following neurotoxins:

MPTP

Mice were injected in the morning of the experimental day with MPTP in 0.9% saline or vehicle alone (single injection, 40 mg/kg, s.c. in the neck, Papers ***I, III, IV*** or 4 x 15 mg/kg, starting in the morning with 2h intervals i.p. Paper ***IV***).

Monkeys were given injections with MPTP (0.2 mg/kg, i.v.) for 13 consecutive days.

Animals were analyzed at different time points after the lesion according to Table 2.

Table 2. Summary of parkinsonian animals models used

Lesion/species	Administration route	Dosage (mg/kg)	Analysis time (h-weeks post-lesion)	Paper
MPTP/mice MPTP/mice	s.c. i.p.	40 mg/kg, single dose 4 x 15mg/kg	4h-12w. p-l 23 d. p-l	I, II, IV IV
MPTP/monkeys	i.v.	13 x 0.2mg/kg, daily	14 w.	IV
6-OHDA/rats	Right mfb	4µg in 2µl sterile water	17 w.	IV

w. weeks, p-l post-lesion, mfb medial forebrain bundle,

6-OHDA

The 6-OHDA lesion was induced by applying the toxin (4 µg) into the medial forebrain bundle of male rats (Paper ***IV***). Thirty min prior to surgery, animals were injected with pargyline (5 mg/kg; monoamine oxidase inhibitor, i.p.) and desipramine (25 mg/kg;

noradrenaline uptake inhibitor, i.p.) to increase the nigrostriatal dopaminergic selectivity of the toxin. Rats were then placed in a stereotaxic frame and each animal received a unilateral injection of 4 µg toxin (in 2µl sterile water with 0.1% ascorbic acid) or vehicle into the right medial forebrain bundle (Bregma (Br) –2.8 mm, 2 mm lateral to the midline (L), and 8.6 mm below the dura (V) according to Paxinos and Watson ((Paxinos and Watson 1986). The 6-OHDA injection was made over a 5 min period using a 5 µl Hamilton syringe. Animals were analyzed at different time points after the lesion according to Table 2.

3.3.2 Tracer, virus delivery and cell proliferation marker

Intracerebroventricular delivery through injections and osmotic pumps

Several substances were delivered intracerebroventricularly by an injection into the right lateral ventricle or via a brain infusion kit at the same coordinates (mouse Br: 0.0 mm, L:1.1 mm , V: 2 mm; rat Br: -2.8 mm, L: 2 mm , V: 8.6 mm) linked with an osmotic pump (Alzet, B&K Universal, Sweden). The following pumps were used (model/delivery time/flow per h): 1003D/3 days/ 1 µL; 1002/14 days/0.25 µL; 2001/7 days/1 µL; 2002/14 days/0.5 µL; 2004/21 days/0.25 µL; 2ML2/14 days/5.0 µL.

Substances for labeling of ependymal cells in vivo (Paper ***I***)

DiI (1,1´-dioctadecyl-6,6´-di-(4-sulfophenyl)-3,3,3´,3´-tetramethylindocarbocyanine) 2.5µl 0.2% w/v (DiI, Molecular Probes) in dimethylsulfoxide (DMSO, Sigma) was injected into the right lateral ventricle. Note! Tissue sections labeled with DiI were not incubated with any solutions containing detergents such as Triton X-100.

Rhodamine-conjugated latex beads (3µl, Lumafluor Inc., FL, USA) diluted 1:50-100 in phosphate buffered saline (PBS).

DiI or Beads labeled cells were counted in the contralateral SNpc to avoid bias by retrograde neuronal label(Doetsch et al. 1999a). Injections were done in the right lateral ventricle.

Substances for retrograde neuronal labelling

Fluorogold (0.3µl of 5% (w/v) in 0.9% saline, Fluorochrome) was injected into the left striatum of the mouse (Br: -0.5 mm, L: -2.0mm V: , 2.4 mm) 48-72 h prior to sacrifice.

eGFP PRV GS518 (enhanced green fluorescent protein expressing pseudorabies virus) was injected stereotaxically with $7.5 \cdot 10^4$ particles of the eGFP into right somatosensory cortex 72 h before the mice were killed. This virus selectively infects and replicates in neurons, passing on to infect higher order neurons within neuronal circuits in a manner strictly dependent on synaptic contact (Carlen et al. 2002).

Alexa594-conjugated cholera toxin B (CTB, Molecular Probes). To exclude neurons labeled through direct retrograde tracing, it was dissolved in 100µL of the pseudorabies virus solution used in the stereotaxic injection, i.e. in a concentration by far exceeding the diffusion of the virus. This tracer is not trans-synaptically transmitted to second order neurons.

5-bromo-2´-deoxyuridine (BrdU, Sigma) was administrated with various regimens as summarized in Table 3 (as following).

Table 3. Summary of the various types of BrdU administration

Admini-stration route	Solution (mg/mL)	Dose (mg/kg)	Duration (hours-weeks)	Survival time (days -weeks after last dose)	Paper
drinking water	1		2-6 w.	0-8 w.	**I**
i.p.	10	65-100 per injection	Single or repeated dose every 2 h for 8-48 h	0-5 w.	**I, III**
i.c.v	50-150 [a]	~ 30–40 per day	Continuous 2, 10, or 21 d.	0 or 3 w. 3 w.	**I II**
i.c.v	1	~ 2 (mouse) ~ 0.04 (rat) per day	Continuous 14 d.	2 d. (mouse) 0 (rat)	**IV**

i.p. intraperitoneal, i.c.v. intracerebroventricular, d. days, w. weeks

[a] BrdU solution was dissolved and prepared for loading the pump as follows: BrdU (Sigma) was added to *at least 2 mL* 0.1M PBS to obtain a final concentration of 150 mg/mL (pH=7.8, pH adjusted with 2N NaOH) and warmed (maximum +50ºC) in a glass jar overnight (>16h) with vortex, to dissolve BrdU. The following day, when the solution was clear, it was sterile

filtered and injected into the osmotic pump kept at +37°C for 2-5 h prior to implantation as described above.

[^{3}H]-thymidine (4 mCi/kg, 6.7 Ci/mmol, NEN) was delivered over three days via osmotic pumps (i.p., Alzet 1003D, see above) and animals were analyzed six weeks later (Papers ***I, II***).

3.3.3 Proliferation inhibitor and PDGF-BB

Ara-C (Papers ***I, IV***) One group of DiI-injected mice (n = 4) received the proliferation inhibitor cytosine-D-arabinofuranoside (Ara-C, Sigma) 4% w/v (40 µg/mL) in 0.9% saline infused on the brain surface for 14 days with an osmotic pump (Alzet 1002, see above) (Paper ***I***).

In rats, the experimental dose of Ara-C was titrated by administering the drug to four groups of rats, either vehicle alone (comprising of BrdU 1 mg/ml and 0.1% rabbit serum albumin (RSA) in PBS), or vehicle plus 0.55 µg/ml, 3.3 µg/ml or 20 µg/ml Ara-C in the pumps. The chosen experimental dose of Ara-C was 10 µg/mL (Paper ***IV***).

PDGF-BB (Paper ***IV***) The PDGF solution was prepared as follows: 10 µg PDGF-BB (Sigma) was dissolved in 10 µL of 1M HAc, and pH was adjusted to 7.4 by adding 10 µL 2N NaOH. A buffer (100mL, 0.01M PBS containing 5 mg/mL BSA and 20 mM Hepes) was added to a total final volume of 400 µL. This solution was further diluted (2.5 – 6 ng/µL) and the final dose was 36-60 ng/day in rodents, i.e. ~1.2 - 2 µg/kg per day in mice and ~120 ng/kg per day in rats. The substance was delivered intracerebroventricularly using osmotic pumps as described above. The cynomolgus monkeys received 2880 ng/day PDGF-BB, i.e. ~ 600 ng/kg per day. Different survival intervals after PDGF-BB were used ranging from 2 days to 7 weeks.

3.4 General Tissue preparation

The adult animals were deeply anesthetized and perfused through the ascending aorta with 50 mL of warm (+37°C) fixative, followed by the same, but ice-cold, fixative for 5 min. The brain was rapidly dissected out and immersed in the same fixative for 90 min and then rinsed

in 10% sucrose in PBS for 24 h. Several fixatives were used for the different experiments: (**1**) For ultrastructural analysis, 4% paraformaldehyde adding 0.25%-0.5% glutaraldehyde, or 3% glutaraldehyde in 0.1 M PBS, pH 7.4 without paraformaldehyde was used as fixative, brains were postfixed for 12h. After cutting the tissue into 100 µm thick vibratome sections, these were postfixed in 1% acetate buffered OsO_4 (Sigma) for 2h at +20°C and dehydrated with 25% ethanol followed by acetone/durcupan (Fluka) over night and then embedded in durcupan. Semithin (1 µm) sections were stained with toluidine blue and ultrathin sections were collected on one-hole carbob-stablized formvar copper grids and contrasted with uranyl acetate (Merck) followed by lead citrate and analyzed in a Philips CM12 or 420 (Eindhoven) electron microscope (EM) (Papers ***I, III***) (**2**) For immunohistochemical analysis in the light microscope brains were fixed at +4ºC with 4% (w/v) paraformaldehyde and 0.4% (v/v) picric acid in 0.4 M PBS, pH 6.9, followed by immersion in the same fixative for 1.5 h and then kept in 10% sucrose overnight, followed immersion in 30% sucrose until brains sunk to the bottom. Sections were cut in a cryostat (14-40 µm thick) and thawed onto pretreated slides of kept free-floating in 30% ethylene glycol with 30% sucrose in 0.1 M PBS as described elsewhere (Chan et al. 1997, Janson and Møller 1992, Walters et al. 1999) (**3**) Brains used for activated caspase-3 and activated caspase-9 analysis were perfused with paraformaldehyde without picric acid. (Paper ***III***) (**4**) For labeling of DNA fragments or performing autoradiography in combination with tyrosine hydroxylase immunohistochemistry, brains were hemisected along the midline and frozen by immersion in isopentane (–45°C) or dipped in 30% sucrose followed by (–70°C) (Papers ***II, III, IV***) After cutting the frozen brains in a cryostat (at -17°C), sections taken to DNA fragmentation assays were immersion-fixed in 4% formalin containing 1.5% methanol for 10 min and kept in 0.1 M PBS with 0.1% NaN_3 until *in situ* end labeling or *in situ* nick translation (Paper ***III***). For autoradiography methods, sections were kept at (–80°C) until incubated with the radioactive compound, see autoradiography below for details about the ligand (Paper ***IV***). Sections from the same brains taken to immunohistochemistry were fixed in 4% (wt/vol) paraformaldehyde/PBS for 10 min. (**5**) For all other immunohistochemical procedures in the non-human primate, the hemisected midbrain was immersion fixed and preserved in 10% buffered formalin, embedded in paraffin wax and sectioned at a thickness of approximately four microns. Before performing immunohistochemistry as described below, these sections were deparaffinised by incubation at +65ºC for 30 min followed by antigen retrieval in 1 x DIVA Decloaker buffer (Biocare

Medical) at +121ºC for 20 min in a pressure cooker (Retriver2100). Slides were rinsed several times in 1x Hot Rinse buffer (Biocare Medical) and in dH_2O water before further processing.

3.5 Histochemistry procedure

3.5.1 Immunohistochemistry and tissue staining methods (Papers *I, II, III, IV*)

For details on antibody concentrations, see the respective papers. Neurons were studied with primary antibodies against: tyrosine hydroxylase, (TH, rabbit polyclonal, Pel-Freeze or Chemicon; mouse monoclonal, Incstar), neuron-specific nuclear protein (NeuN, mouse monoclonal, Chemicon), γ-amino-butyric acid (GABA, gift from O. Shupliakov (Gustafsson et al., 2002)), βIII-tubulin (Tuj-1, mouse monoclonal, BAbCO), Hu (mouse monoclonal, clone 16A11, Monoclonal Antibody Facility, University of Oregon). Glial cells were studied with primary antibodies against: glial fibrillary acidic protein (GFAP, Dako), transferrin (Nordic), macrophage antigen complex-1 (Mac-1. Cedarlane), and ionized calcium binding adaptor molecule 1 (Iba-1, Abcam). Immature precursor/progenitor cells cells were studied with primary antibodies against: Oligodendrocyte lineage transcription factor 2 (Olig2, Santa Cruz), melanoma-associated proteoglycan (NG2, Santa Cruz), doublecortin (dcx, Santa Cruz), SRY (sex determining region Y)-box 2 (Sox2, SantaCruz), nestin (rabbit polyclonal, gift from R.D. McKay) and CRMP-4 (rabbit polyclonal, gift from S. Hockfield, (Minturn et al. 1995). Proliferating cells were studied with primary antibodies against: BrdU (mouse monoclonal, Dako or Boehringer; rat monoclonal, Accurate or Harlan Sera Labs), Ki67 (Immunkemi). Apoptotic cells were studied with primary antibodies against: activated caspase-3 and activated caspase 9 (collaboration with T. Momoi, (Tanaka et al. 2000) and (Fujita et al. 2000) respectively) and antibodies against digoxigenin detecting dUTP-digoxigenin (Intergene). Injected tracers were studied with primary antibodies against: green fluorescent protein (GFP, Chemicon) and fluorogold (FG, Fluorochrome). Before incubation with BrdU antibodies, denaturation of DNA was performed using HCl and 0.025-0,25% pepsin or 2M HCl at +37°C for 30 min.

The secondary antibodies used were linked with the following markers: fluorescein isothiocyanate (FITC), rhodamine, Cy-3, Cy-5, Alexa 555, Alexa 488, 7-amino-4-methylcoumarine-3-acetic acid or biotin, or used as substrate for the avidin-biotin peroxidase reaction (Vector Labs) according to the manufacturer's instruction with 0.03-0.05% 3.3'-diaminobenzidine (DAB) and 0.01-0.3% H_2O_2 as chromagen.

Vascular cells were detected with FITC-conjugated tomato lectin from lycopersicon esculentum (Vector) and biotin-labeled UTP in the DNA fragmentation method below was detected with horseradish peroxidas-conjugated avidin (Vector).

Cell nuclei were counterstained with 0.5% cresyl violet (CV, Nissl stain, Sigma) or Meyer-hematoxylin (Sigma) or stained with the nuclear marker 4´,6-diamidino-2-phenylindole (DAPI) (Sigma). In all immunohistochemical reactions blocking solutions of the same species as the host for the secondary antibodies were used. Endogenous peroxidase activity was blocked by incubation of sections in 0.3% H_2O_2 (Sigma) in all staining procedures using DAB/H_2O_2 as chromagen. Positive and negative controls were performed during all procedures.

3.5.2 Methods detecting DNA fragmentation

To study apoptosis in the midbrain (Paper *III)* , DNA fragmentation was detected in condensed nuclei with *in situ* end labeling, similar to the method known as TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin-labeled nick end labeling), but replacing biotin with digoxigenin (Gavrieli et al. 1992). The immersion-fixed sections were digested in 0.05 M Tris buffer, pH 8.0, containing 1 µg/mL proteinase-K (merck) at +20°C for 5 min. After blocking endogenous peroxidase, terminal deoxynucleotidyl transferase (TdT) with digoxigenin-labeled dUTP (Intergen) and detected as decribed under Immunohistochemistry above.

In situ nick translation detecting single-stranded DNA breaks in condensed nuclei was also used to analyze DNA fragmentation, based on published methods (Meyard et al., 1992; Wijsman et al., 1993). The immersion-fixed sections were digested with 0.005 % pepsin (Sigma) in HCl, pH 2.0, under gentle agitation at +37°C for 5 min, followed by incubation in 0.01 M nucleotides (dATP, dCTP, dGTP and biotin-labeled dUTP) and DNA polymerase-1 (Boehringer). Detection of biotin was done as described above under staining methods above. Both techniques were detected using DAB/H_2O_2 as chromagen.

3.5.3 Methods utilizing radioactive compounds

DAT-binding (Paper ***IV***) Levels of the dopamine transporter (DAT) were determined by receptor-radioligand binding autoradiography using (*E*)-*N*-(3-iodoprop-2-enyl)-2β-

carbomethoxy-3β-(4'-methylphenyl) nortropane (PE2I, specific activity 2000 Ci/mmol (Guilloteau et al., 1998) in the striatum. Sections were incubated for 90 min at +25°C with 50 pM PE21 in pH 7.4 phosphate buffer, the autoradiography method has been described elsewhere (Bezard et al., 2001a). After washing the sections, they were dried and exposed to β-radiation sensitive film (Hyperfilm β-max, Amersham) in X-ray cassettes for 2 days. Densitometric analysis was performed with an image analysis system (Image ProPlus). Three sections per animal at rostral and caudal parts of the caudate-putamen were analyzed by an examiner blinded to the experimental conditions. Data was expressed as fmol/mg of tissue equivalent.

Preproenkephalin mRNA expression (Papers ***IV***) Sections were hybridized with a [^{35}S]-dATP-labelled probe as described earlier (Bezard et al., 2001b) at +42°C for 18 h and exposed to β-radiation sensitive film (Hyperfilm β-max, Amersham) for 14 days at +4°C. Data was expressed as optical densities in the different quadrants of the right caudal caudate and putamen.

[^{3}H]-thymidine (Papers ***I*** and ***II***) Sections were dipped in NTB2 emulsion (Kodak) and exposed for 4 weeks in the dark at +4 or -20°C, developed in D-19 (Kodak), fixed, and counterstained with CV. Cells with more than 20 silver grains over the nucleus were considered labeled cells.

SPECT-analysis (Paper ***IV***) All non-human primates were evaluated with an *in vivo* method to assess DAT-binding in the nerve terminal regions of the forebrain with a Single Photon Emission Computerized Tomography (SPECT, Siemens) apparatus. Animals were their own controls, exmined before and 4 weeks after MPTP as well as 4 weeks after initiation of PDGF-BB treatment. [^{99m}Tc] 2beta-((N,N'-bis(2-mercaptoethyl) ethylene diamino)methyl), 3beta-(4-chlorophenyl) tropane) (TRODAT, bolus 10 mCi, intravenous (i.v)).

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3.6 Photoconversion (Paper ***I***)

Photoconversion of DiI was performed as described elsewhere (Johansson et al. 1999).The DiI-labeled sections were first incubated in 1% H_2O_2 in 0.1 M Tris-HCL (pH=8.2) 10–15min, wash sections in Tris-HCL buffer and sections were pre–incubated for 45 min–60 min at +4°C in the dark in DAB (1.5mg DAB/ml of Tris, pH 8.2, filtered with an acrodic (0.45µm

sterile syringe filter, Gelman sciences, Arbor). Following incubation, the sections were rinsed again with Tris, and flattened onto a depression slide (which can be made using a 'Pap-Pen'), after which 1-3 drops of the DAB solution was added to cover the area of interest, which was then located and irradiated using an Olympus Inverted Research Microscope with epifluorescence and a 100W mercury bulb using a rhodamine filter cube under the desired objective (10X, objective) for 1.5 h, the DAB solution was changed after 30 min of irradiation and then replaced with fresh, cold, filtered DAB. The progress of the reaction was monitored by switching between fluorescent and white light until a dense, brown DAB product was visualized.

3.7 Cell culture (Paper ***I***)

Tissue dissociation and culture conditions were as described (Johansson et al. 1999) except that both epidermal growth factor and fibroblast growth factor-2 were used 20 ng/ml in the medium to generate neurospheres. Differentiation of the neurospheres was induced by plating on fibronectin/ornithin-coated slides. After one week, neurons and glia were detected with immunohistochemistry.

3.8 Stereological Analysis of Cell Numbers

All analysis was performed on coded slides, in order to secure that the examiner was blinded to the experimental conditions. Systematically sampled 20-40µm thick coronal cryostat sections were obtained from the entire SNpc.

Every sixth section (or every section in the analysis of BrdU-positive nigral cells) was stained with immunohistochemical markers and the nucleolus was counterstained with cresyl violet. Stereological analysis was performed using an Olympus BH2 microscope with a motorized X-Y stage linked to a computer-assisted stereological system. This is comprised of a color video camera (CCD-Iris, Sony), a PC with a high resolution monitor, a microcator (VRZ 401, Heidenhain) and the Castgrid software (Olympus, Albertslund, Denmark) (Papers ***I,II, III and IV***)

On each section through the length of the region, the SNpc was delineated at low magnification (4x) as shown elsewhere (Evans et al. 2004, McCormack et al. 2004). The ventral tegmental area (A10) and the retrorubral field (A8) were excluded. From a random start position, a counting frame was superimposed on the image and a sampling region was chosen by the computer, dissected with a 100x oil immersion lens with a high numerical aperture (1.4) into thin focal planes (0.5 µm). Cells were counted according to the rules of the optical disector using the nucleolus (neuron) or upper limit of the cell nucleus (other cells) as the sampling structure (Janson and Moller 1993, West et al. 1991).

The nucleolus provides a precise neuronal marker, since it is small and dopaminergic neurons in substantia nigra only have one nucleolus per nucleus. To avoid bias from poor antibody penetration or due to knife artefacts, a randomly selected animal from each experiment was analyzed to detect if any parts of the section along the section thickness displayed an abnormal distribution of cells (fewer cells at the surface (< 2 µm below the cover glass) and at the bottom of the slide were found as a rule). The height of the sampling volume along the z-axis was selected from the frequency distribution of cells in the part of the section that had a normal distribution of sampled cells (Walters et al., 1999; McCormack et al., 2004). The total cell number of several different neuronal, glial and progenitor cell populations was estimated using the optical fractionator technique determining the coefficient of error (CE) of each estimate (Janson and Moller 1993, West et al. 1991). The volume-estimates of cells using the vertical rotator (Janson and Moller 1993)(Janson and Moller, 1993, Jensen and Gundersen, 1993) was performed in vertical sections after random rotation around the dorso-ventral midbrain axis (Baddeley et al., 1986).

3.9 Behavioral tests (Paper *IV*)

Rotation of 6-OH DA-lesioned rats

The imbalance between the partially lesioned (right) and intact (left) dopaminergic systems was analyzed as the rats rotated after injection of 0.05 mg/kg sc apomorphine (contraversive turns) and 5 mg/kg i.p. amphetamine (ipsiversive turns)(Ungerstedt 1968). Animals displaying rotations when challenged with amphetamine, but not with apomorphine, were considered partially lesioned (Moore et al., 2001). The analysis of the duration and intensity

of the rotational behavior during 1 h was examined in automated rotometers and repeated weekly in order to follow signs of restoration in behavior.

Parkinsonism in MPTP-lesioned non-human primates Activity, parkinsonian disability and dyskinesia were assessed in 2 h periods weekly. Activity was assessed with infrared detectors. The degree of lesion was studied with a validated parkinsonian macaque clinical scale analyzing tremor, variations in general activity, body posture, vocalization, freezing, frequency of arm movements and rigidity. Parkinsonian disability and dyskinesia after PDGF-BB treatment were scored by an examiner blinded to treatment. The scales on parkinsonism are designed for videotaped behavior and include estimates on range of movement, bradykinesia, body posture and tremor.

3.10 Statistics (Paper *I, III, IV*)

Data are expressed as mean $\pm$ S.E.M. or mean (Coefficient of variation, C.V.). When only two groups were compared, Student's T-test or the non-parametric Mann-Whitney U-test was used. Bonferroni's procedure to balance for type I error was done after multiple comparisons in the same animal. Multiple comparisons were performed with ANOVA or non-parametric tests such as Kruskall-Wallis with appropriate post-hoc tests. Correlations were analyzed with Spearman's correlation test.

in combination with CV staining to demonstrate Nissl substance in the cytoplasm of labeled nuclei. In order to firmly establish the neuronal phenotype of the adult-born cells we used electron microscopy. We could establish the presence of [^{3}H]-thymidine labelled neurons with synaptic contact on its soma, thus proving strong evidence that the newly generated cells were not glia. Taken together, the data exclude the possibility that nuclei from glia cells close to neurons were misinterpreted as neuronal labeling.

4.2. Methodological discrepancies may explain conflicting results on adult neurogenesis in substantia nigra

Variations in the labeling efficiency of delivered BrdU are often pronounced, and methodological differences may explain discrepancies in adult mammalian neurogenesis in the brain outside the widely accepted neurogenic regions, i.e. hippocampus and olfactory bulb/subventricular zone. When smaller nerve cell populations have been suggested to have a neurogenic potential, under both physiological and injury-induced conditions, the results have been conflicting as summarized by Gould (2007). Not only the above listed factors, but also differences in duration and dosage, as well as administration pathway and survival time, may result in variable results, but also the animal housing conditions, as well as the method employed to process the tissue, the sampling of sections, the visualisation of the proliferation marker, the definition of brain regions and the counting of BrdU-labelled neurons (Evans et al. 2004, Kee et al. 2002). This may explain negative reports from adult brain regions such as substantia nigra (Frielingsdorf et al. 2004, Mohapel et al. 2005), where others have presented evidence for neurogenesis (Peng et al. 2008, Shan et al. 2006, Van Kampen and Eckman 2006, Van Kampen and Robertson 2005, Zhao et al. 2003).

In Paper ***II***, we infused a high dose of BrdU for three weeks into the brain and detected the signal in the adult SNpc after an additional period of three weeks. A control experiment was carried out using systemic continuous infusion of [^{3}H]-thymidine. We also studied, if the high dose of nucleotide analogue altered the total number of nigral neurons showing immunoreactivity for the dopaminergic marker TH. There are studies employing BrdU administration into the cerebral ventricles reporting that BrdU labeled TH-positive neurons cannot be detected in substantia nigra. However, those studies only employed 50% (Frielingsdorf et al. 2004) or <1 % (Mohapel et al. 2005) of the BrdU concentration used in our report (Zhao et al. 2003). Importantly, the first **paper *I*** analyzed BrdU-treated animals

without an additional three weeks survival, which was used here to allow maturation of the dividing cells into neurons at detectable levels. Interestingly, both Frielingsdorf et al. (Frielingsdorf et al. 2004) and Zhao et al. (Zhao et al. 2003) reported similar numbers of labelled nigral non-neuronal cells, underlining the differential kinetics for neuronal and non-neuronal BrdU uptake. Besides differences in the BrdU detection method outlined above, other discrepancies including the environmental conditions for the experimental animals (single cages as opposed to our socially enriched environment) could possibly explain our conflicting results.

Our data support adult nigal neurogenesis at a low rate is based on a special method to deliver BrdU at high concentrations (150 mg/mL, i.c.v.) into the adult mouse brain. The dosage and duration of BrdU appear critical, since exposure to low doses did not result in a robust label (Paper ***I***). To avoid labelling cells with a fast turnover, the mice were allowed to survive three weeks after ending the BrdU delivery. Midbrain sections were processed for TH immunohistochemistry, post-fixed with 4% paraformaldehyde, to protect the TH staining against deterioration by 2N HCl and 0.025% pepsin used for denaturation of the DNA, followed by immunolabelling of nuclear BrdU in single stranded DNA. Double-labelled cells were analyzed in a confocal laser microscope and showed segmented nuclear BrdU label surrounded by TH-immunoreactive cytoplasm, never displaying apoptotic morphological features. The protocol was well tolerated by the mice and not found to be toxic for the region studied, i.e. did not alter the total number of nigral neurons.

A similar BrdU staining configuration in neuronal nuclei was found by another research group(Sauerzweig et al. 2009) with long survival (>3 weeks) after BrdU administration. In contrast, when analyzed only a few days after systemic administration, Sauerzweig and colleagues (Sauerzweig et al. 2009) demonstrated that the BrdU label filled the entire nucleus homogenously in the majority of labelled hippocampal dentate gyrus neurons. They suggested that the signal of the incorporated BrdU changed over time from a *filled* to a *segmented* and finally to a *punctuated* staining pattern. It could be interpreted that during early S-phase, replicating DNA is distributed throughout the nucleoplasm, excluding the nucleolus, resulting in a BrdU label that fills the entire nucleus. As the cell matures, replication euchromatin decreases and is replaced by heterochromatin, showing an interconnecting *segmented* pattern, the sites of DNA replication becoming large in size and fewer in number. Toward the end of maturation, the small site of DNA replication (BrdU label) will instead have a *punctuated* appearance.

In paper ***I,*** quantification revealed a few scattered TH+ neurons with BrdU+ nuclei when analyzed three-dimensionally in the confocal laser scanning microscope. In general, labeled nerve cells were detected only after prolonged BrdU. A single BrdU injection only rendered *one* labeled TH+/BrdU+ neuron 5 weeks later in five analyzed mice. Similarly, when [^{3}H]-thymidine was used as an alternative method to demonstrate neurogenesis in substantia nigra, a large number of sampling sites throughout the brain region of interest was needed to detect the newly generated nigral neurons. But since it was difficult to determine a limit, where nuclei located deeper in the section contribute to the radioactive label on top of the section, a different approach for quantitation of tritiated label using the physical disector in a few micrometer thin sections in recommended (Evans et al. 2004).

4.3 Neural stem cells in the midbrain of the adult mouse

Neural stem cells lining the ventricular system of the adult rodent give rise to olfactory bulb interneurons and have been found to reside both in the ependymal and subependymal layer of the lateral ventricle wall (Doetsch et al. 1999b, Johansson et al. 1999). To test whether or not cells lining the ventricular extension in the midbrain have stem cell properties, we established cultures from the third ventricular recess and the cerebral aqueduct after an i.c.v. injection of DiI (Johansson et al. 1999) or rhodamine-conjugated latex beads (Doetsch et al. 1999b) in 12-week-old mice (n = 6). That the DiI uptake was restricted to the ependymal layer was confirmed by EM after photoconversion of DiI in the injected specimens (n = 2). Labeled cells proliferated *in vitro* to form clonal aggregates, i.e. neurospheres. When passaged, single cells generated new neurospheres which, when induced to differentiate, generated both neurons and glia, indicating that they were neural stem cells. However, the cells did not spontaneously acquire a dopaminergic phenotype, which indicates the presence of signals for phenotypic differentiation *in vivo*.

4.4 Origin of adult-born neurons in the substantia nigra

Neural stem cells along the cerebroventricular extension in the midbrain are likely to give rise to the observed new neurons in the adult substantia nigra. Six hours after an injection of DiI into the lateral ventricle, the label was restricted to the ependymal layer of the midbrain aqueduct. A few days later, DiI-labeled cells were also seen outside the ependymal layer. Four days later, animals receiving BrdU through drinking water, proliferation of cells lining the

aqueduct was evident. DiI-labeled BrdU+ cells with a morphology associated with *migratory* cells were seen along the ventral midline. At seven days after DiI, a DiI-labeled cell was observed to co-label with the immature neuronal marker CRMP-4, and some nerve cells in substantia nigra were both immature cell markers nestin and TH. Starting about ten days after ependymal DiI injection, we observed first DiI+/TH+ neuron in the SNpc, indicating similar neuronal migration kinetics as found in the rostral migratory stream. This argues against the possibility that the neurons had been retrogradely labeled from the injection site, which is a fast process. The neuronal identity of the DiI-labeled TH+ cells was supported by NeuN labeling. Similar results were obtained after i.c.v. injection of rhodamine-conjugated latex beads. The number of DiI+/TH+ neurons was reduced in animals receiving BrdU in the drinking water for the first 42 days after DiI (open circles, $n = 7$). The number of DiI-labeled TH+ neurons in the contralateral substantia nigra increased in a linear fashion over time. Importantly, double-labeled neurons were observed in the rostral SNpc, but never in the most caudal part, where sparse contralateral nigrostriatal projections have been observed (Morgan et al. 1986).

We have attempted to verity the link between proliferative precursor cells and appearance of DiI labeled neurons in SNpc. Thus, the specificity of DiI in SNpc was confirmed by giving an intracranial infusion of the anti-mitotic agent Ara-C (see also paper IV, where a lower concentration of Ara-C was infused into rats). In animals that received this proliferation inhibito, no DiI-labeled TH+ neurons in the contralateral substantia nigra were found 3 weeks later, proving that the DiI labeling paradigm largely lables cells that are generated by cell proliferation, and thus strongly argueing against retrograde axonal transport. Based on the steady increase of DiI-labeled TH+ cells, we estimated that 20 new neurons were added each day (≈0.17% of the total nigral population). This estimate is substantially higher than that suggested by nucleotide analogue labeling in paper I and none of the used BrdU administration protocols labeled all DiI cells. A marked decrease in the number of DiI-labeled TH+ neurons was seen in animals which received a six-week-BrdU administration after DiI. The most likely explanations for this discrepancy is toxicity in proliferating cell populations or inefficient labeling of dividing cells with BrdU. The issue of incomplete labeling of dividing cells in rodent brain has been the focus of other studies. When injecting high doses of BrdU into rats, a substantially increased number of cells were labeled in the adult rat dentate gyrus, compared to other studies using low doses (Cameron and McKay 2001).

4.5 Newly generated neurons project to the striatum and integrate into synaptic circuits

We have tested, if newly generated TH labeled neurons project to the striatum. We injected the retrogradely transported axonal tracer fluorogold (FG) into the left striatum of animals that had received an i.c.v. DiI injection to label ependymal cells 4–8 weeks (but not 2 weeks) earlier. We estimated that ≈10% of the DiI-labeled nerve cells in the substantia nigra also contained fluorogold. Moreover, in animals (n = 8) that had received BrdU 2 months (but not 3 weeks) earlier, we found a few fluorogold labeled nigral neurons that had incorporated the nucleotide analogue, suggesting that adult new-born nigral neurons project axons to the striatum. We next analyzed whether or not the newly generated neurons integrated into the synaptic circuitry by injecting eGFP (green fluorescent protein)-expressing pseudorabies virus PRV GS518 into the somatosensory cortex of adult mice 1 month after receiving BrdU in the drinking water for 3 weeks. When directly injected into the brain, this virus selectively infects and replicates in neuron and is actively transported along axons and dendrites to synapses, where is passes on to infect higher order neurons within a circuit (Enquist et al. 1998). In contrast, the axonal tracer cholera toxin B (CTB) is not transynaptically transmitted to second order neurons. The colocalization of eGFP in BrdUrd+ neurons without CTB contralateral to the injection suggested that the adult-born neurons in the substantia nigra participated in defined multisynapse network connecting with the cortex.

4.6 Increased neurogenesis after a partial lesion

Several studies have demonstrated lesion-induced effects on neurogenesis in other brain regions (Kornack and Rakic 2001, Magavi et al. 2000). To determine whether or not a specific lesion to the substantia nigra has any effect on the rate of nigral neurogenesis, MPTP (40mg/kg, s.c.) was administered to 10-week old C57BL/6 mice to selectively cause a partial lesion in SNpc (Chan et al. 1997). A 2-fold increase of BrdU incorporation in TH+ nigral neurons was observed 3 weeks after the lesion (Fig. 4*a*). Mice received BrdU 10 days and 3 weeks by i.c.v. infusion. For analysis of DiI-labelled nigra neuron after MPTP, mice received DiI injection and BrdU delivery at the same time. The results are summarized in paper I.

4.7 Neuronal apoptosis through caspase activation is accompanied by an increase in neuronal nestin expression in the MPTP mouse model

To test the hypothesis that neurons die by apoptosis in animal models of PD, we exposed mice to a selective, toxic regimen which led to neuronal apoptosis, peaking three days post-MPTP (40 mg/kg, s.c.). The time course of nigral *in situ* end labeling and *in situ* nick translation corresponded to the estimated neuronal loss in MPTP-treated mice, indicating that apoptosis is slower than expected and that nigral neuronal death through apoptosis spans a time period of several days. We also wanted to assess the parallel dynamic changes in nigral progenitor cell populations. The experimental design is summarized in Fig. 3.

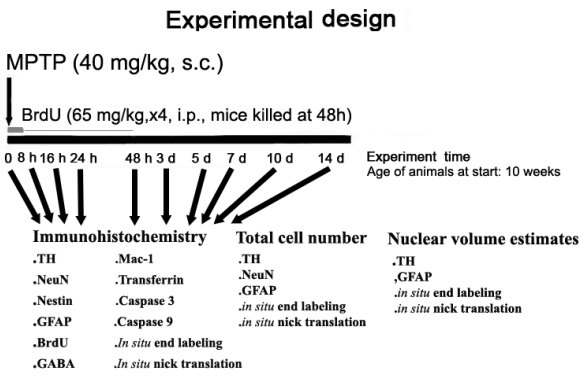

Fig. 3. Experimental design of paper *III*. The design allows an analysis of both neurodegenerative and neurorestorative events in the acute phase after a single lesion. In order to understand the dynamic changes in various nigral cell populations, it is important to use a simple model avoiding multiple MPTP injections.

The neuronal identity of the MPTP-induced apoptosis was confirmed by i) confocal microscopy of *in situ* end labeled cells, either co-labeling with TH, or NeuN, ii) nuclear volume estimates of *in situ* nick translated cells and *in situ* end labeled cells (non-apoptotic neurons > apoptotic nuclei > glial cell nuclei) and iii) ultrastructural analysis. The number of neurons lost per time interval and the total number of apoptotic cell nuclei estimated with the two techniques indicated that the prevailing mechanism of neuronal death was apoptosis (correlation coefficient 0.92), mediated by caspase activation at two days after neurotoxin. Condensed nuclei with fragmented DNA preceded loss of neuronal markers such as TH and NeuN, which disappeared during the apoptotic process of further nuclear condensation.

Caspase-9 activation was only observed at day 2 post-MPTP, whereas caspase-3 activation was observed both at day 2 and 3 post-MPTP. We interpret the rare occurrence of activated caspase in NeuN+ nigral neurons to indicate the small time window for caspase expression. In separate animals the correlation between TH+ and NeuN+ nigral neurons was studied, indicating that neurons do not only lose TH expression but also NeuN+ and neuronal Nissl substance stained with cresyl violet as they undergo apoptosis.

Parallel to the early neurodegenerative events, increases in nigral cell populations expressing the intermediate filament nestin were observed. The majority of these cells had a glial morphology, sometimes co-expressing GFAP and showing nuclear incorporation of nuclear BrdU, which was incorporated two days before the mice were killed, i.e. to soon after administration and too low concentrations of the thymidine analogue to allow neuronal BrdU incorporation to be observed. Another, distinct subgroup of the nestin-immunoreactive cells displayed a neuronal, bipolar morphology. Importantly, only cells in blood vessels and a few nestin-labeled progenitor cells showed nuclear incorporation of the thymidine analogue BrdU at 2 days after MPTP. Two distinct types of nestin+ cells were found in the adult SNpc, in accordance with earlier reports on both neuronal (Lie et al. 2002, Lu et al. 2005, Tonchev et al. 2003, Zhao et al. 2003) and glial (Clarke et al. 1994, Frisen et al. 1995) precursor cells expressing this immature intermediate filament. We interpret that the rare occurrence of these neurons is related to a narrow window of coexpression of nestin and TH (Lendahl et al. 1990, Steinert and Liem 1990). A neuronal destiny for such nestin+ nigral neurons is further supported by the findings of Lie and colleagues(Lie et al. 2002), where nestin+ cells from the adult SNpc were shown to incorporate BrdU and to differentiate into neurons when transplanted into the hilar region of hippocampus.

4.8 Restorative effects of platelet derived growth factor-BB in models of Parkinson's disease

The PDGF family comprises growth factors that act via tyrosine kinase receptors (Mori et al. 1993). The PDGFs stimulate various cellular functions such a proliferation, growth and differentiation and has been shown to increase survival of dopaminergic cells *in vitro,*

suggesting that *in vivo* administration could be beneficial in PD (Pietz et al. 1996). PDGFs promote the proliferation of glial precursor cells and are possibly important to maintain a pool of neural and glial precursor cells in the brain (Erlandsson et al. 2001, Jackson et al. 2006).

It has been suggested that stem/progenitor cells present in the niche in the SVZ are dependent on a dopaminergic input to maintain a normal cellular turnover (Baker et al., 2004). To address whether a potential effect of PDGF on an increased cell proliferation in e.g. the SVZ can influence the dopaminergic system, offers an alternative strategy to restore function in Parkinson's disease. We investigated the effects of introcerebroventricular (i.c.v.) infusion of PDGF in PD models in rodents (36-60 ng/day, 7-14 days) and non-human primates (2880 ng/day, 14 days). A significant increase in cell proliferation was seen in the ventricular wall and striatum in parkinsoninan **mice**. These proliferative cells were labeled by BrdU, co-labeled with chondroitin sulfate proteoglycan 4 (NG2), double doublecortin (Dcx) and tomato lectin, indicating proliferation of oligodendrocytic and neuronal precursor cells, as well as vascular cells, respectively. Moreover, in MPTP treated **monkeys**, PDGF-BB improved parkinsonian disability, increased DA transporter-binding and normalized striatal pre-pro-enkephalin mRNA expression. The effects on dopaminergic neurons and functional recovery in **rats** could be blocked by coinfusion with a proliferation inhibitor, Ara-C, providing a link between the proliferative and anti-parkinsonian effects. Based on the data, we consider PDGF-BB a clinical candidate drug, and a reversal of an impaired proliferation in a neurogenic niche as an alternative strategy to restore function in Parkinson's disease. Whether this proliferation of progenitors is directly linked with a neurogenic response in substantia nigra remains to be shown. Again, the low dose of BrdU used and the short survival time after administration did not allow sufficient labeling of nigral neurons with the thymidine analogue (see experimental design for BrdU administration in mice, Fig. 4).

5 CONCLUSIONS AND FUTURE PROSPECTS

This thesis has focused on the study of Parkinsonian animal models. The aim has been to test the hypothesis that new dopaminergic neurons are generated in the adult substantia nigra pars compacta under physiological conditions. If such an endogenous neuronal replacement mechanism is present, neuroregenerative approaches could yield promising new therapies for PD patients in the future. Importantly, the newly generated dopaminegic projection neurons integrate into neuronal circuits. Our experiments further show that cell loss may be, at least partially, compensated for by new neurons being continuously born from stem cells in the adult rodent brain suggesting that the rate of neurogenesis is increased after lesion. Interestingly, several research reports have confirmed our findings of neurogenesis in the adult mammalian substantia nigra. We consider methodological differences as the most probable reason why other researchers did not observe the low rate of neurogenesis in this brain region.

The causes of neuronal death in PD is still unknown, several disease mechanisms, both genetical and environmental, have been suggested. We critically assessed the apoptotic nature of the MPTP-induced neuronal death and found that it is mediated by caspase-9-dependent caspase-3 activation accompanied by an increase in neuronal nestin expression, using different neuronal markers, bright field light, confocal laser scanning and electron microscopy. The main objective was to shed light on the complex dynamic changes in the acute phase after a partial dopaminergic lesion, which also stimulates regenerative responses that may be important clinically. We do not know when the nigral nerve cells start to die in Parkinson's Disease, but it is known that there is a progressive cell loss after onset of the disease. Thus it could be of interest to stimulate the neuronal progenitors present in the tissue, which were found to respond to the injury.

The findings reported here could have profound implications for the treatment of PD. Indeed, the loss of primarily one discrete cell population of cells makes PD a prime candidate for such neuroregenerative therapy. On the other hand, we cannot exclude that PD is a disease of the neurogenic niche as a whole, which could explain symptoms also from other brain regions. In fact, PD may be, at least in some cases, a disorder reflecting a shift in the balance between neurogenesis and neuronal death. The recent identification of ongoing neurogenesis in the adult mammalian CNS presents the intriguing possibilities of utilizing endogenous progenitor

cells as a novel source for cell replacement strategies. PDGF-BB was found to influence the neurogenic niche in the subventricular zone and lead to a proliferation dependent restoration of nigrostriatal structures and function. Based on the current data, we consider plateletderived growth factor-BB a strong clinical candidate drug, and a reversal of an impaired proliferation in a neurogenic niche as an alternative strategy to restore function in Parkinson's disease.

To further develop a successful regenerative therapy for the injured brain, we need to more precisely understand the comprehensive mechanisms regulating adult neurogenesis. This may provide a blueprint to help developing strategies to repair the damaged brain after injury or in various neurological diseases (Duan et al. 2008).

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