Neuroprotection and Neurorestoration in the MPTP Model for Parkinson's Disease

Dissertation

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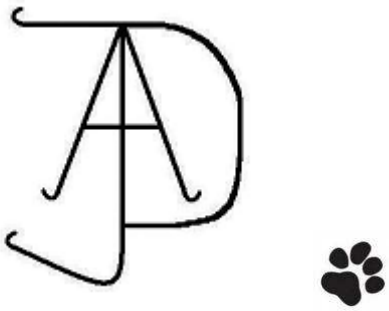
~ Dedicated to my lovely husband ~



«Life is gorgeous with you»

I hereby declare that the thesis „Neuroprotection and Neurorestoration in the MPTP Model for Parkinson's Disease" has been written independently and by no other sources than quoted.

Anja Drinkut

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℃	Degree centigrade
6-OHDA	6-Hydroxydopamine
A30PαSyn	(Thy1)-h[A30P]α-syncuclein transgenic
AAV	Adeno-associated virus
ABC	Avidin-Biotin complex
ACh	Acetylcholine
AD	Alzheimer's disease
AdV	Adenovirus
ALS	Amyotrophic lateral sclerosis
APS	Ammonium persulfate
ARTN	Artemin
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt
BDNF	Brain-derived neurotrophic factor
bp	Base pairs
BSA	Bovine serum albumin
CAT	Peroxisome-located catalase
CBA	Chicken ß-actin
cDNA	Copy-DNA
CDNF	Conserved DA neurotrophic factor
CHAPS	3[(3Cholamidopropyl)dimethylammonio]-propanesulfonic acid
CMV	Cytomegalovirus
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CP	*Caudate putamen*
CREB	cAMP response element-binding protein
DA	Dopamine
DAB	3,3'-Diaminobenzidine
DABCO	1,4-Diazobicyclo-[2.2.2]-octane
DAT	Dopamine transporter
DBS	Deep brain stimulation
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOPAC	3,4-Dihydroxybenyoic acid
DTT	Dithiothreitol
DUB	De-ubiquitinating
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGFP	Enhanced green fluorescent protein
ERK	Signal-regulated kinase
EtOH	Ethanol
FCS	Fetal calf serum
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFR	GPI-linked GDNF family receptor
GFLs	GDNF family ligands
GP	*Globus pallidus*
GPe	*Globus pallidus externum*
GPi	*Globus pallidus internum*
GPI	Glycosyl phosphatidylinositol
GSH	Glutathione
hAADC	Human aromatic l-amino acid decarboxylase
HD	Huntington's disease
HEK293	Human embryonic kidney 293
HEPES	N-2-hydroxyethylpiperazin-N'-2-ethansulfonic acid
HPLC	High pressure liquid chromatography
HRP	horseradish peroxidase
HSV	Herpes simplex virus
HVA	Homovanillic acid
Iba-1	Ionized calcium binding adaptor molecule-1
IGF	Insuline-like growth factor
IL	Interleukine
i.p.	Intraperitoneal
kDa	Kilo Dalton
LB	Luria broth
LBs	Lewy bodies
L-DOPA	1-3,4-Dihydroxyphenylalanine
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
LV	Lentivirus
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAO-B	Monoamine oxidase-B
MAPK	Mitogen-activated protein

	kinase
MEN2B	Multiple endocrine neoplasia type B
MPP^+	1-Methyl-4-phenylpyridinium
MPPP	1-Methyl-4-phenyl-4-propionpiperidine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple Sclerosis
MSA	Multiple system atrophy
NaCl	Sodium chloride
NBT	Nitro blue tetrazolium chloride
NCAM	Neural cell adhesion molecule
NDD	Neurodegenerative diseases
NGF	Nerve growth factor
NGS	Normal goat serum
NP-40	Nonyl phenoxylpolyethoxylethanol
NRTN	Neurturin
NSP	Nigrostriatal projection
NT-3	Neurotrophin-3
OD	Optical density
o/n	Over night
p75NTR	p75 Neurotrophin receptor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PK	Proteinase K
PKC	Protein kinase C
PP	Type-2A- protein phosphatases
PSPN	Persephin
RET	Rearranged during transfection
rh	Recombinant human
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RSV	Rous sarcoma virus
RT	Room temperature
SDS	Sodium dodecylsulfate
SN	*Substantia nigra*
SNpc	*Substantia nigra pars compacta*
SNpr	*Substantia nigra pars reticulata*
SOC	Super optimal broth with catabolite repression medium
SOD	Superoxide dismutase
STN	Subthalamic nucleus
SYN	Synapsin
TAE	Tris-acetate buffer
TB	Transcription blocking element
TBS	Tris-buffered saline
TBS-T	TBS-tween
TE	Tris-buffered EDTA
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)-aminomethan
Trk	Tyrosine kinase
TU	Transducing units
Ub	Ubiquitin
UCH-L1	Ubiquitin carboxy-terminal hydrolase
UV	Ultra violet
VMAT	Vesicular monoamine transporter
Wt	Wildtype

Parkinson's disease (PD) is the most common neurodegenerative movement disorder of the *basal ganglia* and is characterized by a progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). The glial cell line-derived neurotrophic factor (GDNF) has been identified in preclinical experiments as an important differentiation and survival factor for dopaminergic neurons of the midbrain, yet its application in clinical trials has shown inconsistent efficacy. Current gene therapies delivering neurotrophic factors via neuronal expression may not fulfil essential safety criteria for more elaborate strategies with higher dosages or younger patients. Given that neurotrophic factors are potent modulators of neuronal physiology and thus may potentially provoke unwanted side effects if present in brain areas not affected by PD, it seems preferable to restrict their impact to the immediate vicinity of the site of lesion. In this study it was investigated, whether adeno-associated virus (AAV)-5 vectors injected in low and high titre concentrations into the *striatum* of C57Bl/6-J mice leading to either neuronal or astrocytic production of GDNF have different effects on protective and neuroregenerative efficacy in the 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Unilateral striatal vector application resulted in delivery of bio-functional GDNF to the *striatum* and SN of both brain hemispheres if GDNF was expressed in neurons, indicating distribution to sites far remote from the vector application. In contrast, effects of GDNF were restricted to the injected hemisphere by expression in astrocytes. Astrocytic GDNF expression was neuroprotective at low vector dosage thus representing a safe alternative to current gene therapeutic strategies for PD.

It has been claimed that higher concentrations of GDNF may also signal through receptors other than the prototypical rearranged during transfection (RET) and glycosyl phosphatidylinositol–linked GDNF family (GFR)α receptors. However, using mice with a tissue-selective ablation of the gene encoding *Ret* (*DAT-Ret*$^{lx/lx}$ mice) no alternative to an intact GDNF-RET-mediated signalling in the nigrostriatal system to awaken the protective potential of GDNF after MPTP lesion was found.

1 Summary

1.1 Astrocytic versus neuronal expression of GDNF: implications for gene therapy

PD is the most common neurodegenerative movement disorder of the *basal ganglia*, affecting about 2% of the population over the age of 60. Progressive loss of dopaminergic neurons in the SNpc is the main characteristic of PD and leads to the major clinical abnormalities. GDNF has been identified in preclinical experiments as an important differentiation and survival factor for dopaminergic neurons of the midbrain. The encouraging data from these experiments and the potential for therapeutic applications led to clinical trials, which, however, have shown inconsistent efficacy; possibly related to insufficient putamenal distribution of GDNF. Following another hypothesis, the patients participating in these clinical studies likely had too little of their nigrostriatal dopaminergic tract intact to induce therapeutic effects of GDNF. Effective GDNF delivery to the nigrostriatal system combined with restriction of its impact to the immediate vicinity of the site of lesion would be indeed an attractive clinical option and reduce safety concerns, especially if future Phase II trials might be performed in patients at an earlier stage of PD.

Therefore it will be analyzed if cell-specific GDNF expression, with either selective astrocytic or neuronal production of GDNF, delivers a safe and efficient supply of the nigrostriatal system in an experimental mouse model of PD. MPTP induces a syndrome in humans almost indistinguishable from PD, thus making it ideal to study PD in animal models.

In a first step different systems were characterized, which allowed to distinguish between GDNF produced in astrocytes or neurons by unilateral injection of the cell-specific viral constructs AAV-5 glial fibrillary acidic protein (GFAP) GDNF (transducing astrocytes) and AAV-5 synapsin (SYN) GDNF (transducing neurons) in the *striatum* of C57Bl/6-J mice. Neuronal and astrocytic production of GDNF mediated by AAV-5 resulted in broad long-term expression of GDNF in the *striatum*, but transport of GDNF to the SN was much more efficient if GDNF was produced by neurons.

As confirmed by multiple parameters like immunohistochemistry, nigral dopaminergic neuron numbers, striatal fibre density and dopamine (DA) content it was found that unilateral striatal vector application resulted in the delivery of bio-functional GDNF to the *striatum* and *substantia nigra* of both brain hemispheres if GDNF was expressed in neurons, indicating distribution to sites far remote from the vector application. In contrast, GDNF delivery was restricted to the injected hemisphere by expression in astrocytes. Astrocytic GDNF expression was bio-active at low vector dosage and thus represents a safe alternative to current gene therapeutic strategies to treat PD.

1.2 Ret signalling: the basic requirement for GDNF-mediated protection against MPTP toxicity

GDNF classically signals through a two-component receptor complex consisting of RET and GFRα. It has already been observed in our laboratory that endogenous GDNF-mediated RET signalling has no influence on the survival of dopaminergic neurons in the MPTP model of PD, but rather facilitates the regeneration of dopaminergic axon terminals in the *striatum* in longitudinal studies. The predictive value of these previous results is limited by their dependence on physiological GDNF concentrations, whereas under treatment conditions several times higher concentrations may be achieved.

It has been claimed that GDNF at higher concentrations may also signal through other than the prototypical RET and GFRα receptors. Neural cell adhesion molecule (NCAM) has been proposed as an alternative signalling receptor for GDNF and also other RET-independent GDNF signalling pathways have been described.

Tyrosine kinases like RET signal through the mitogen-activated protein kinase (MAPK) and/or the phosphatidylinositol 3-kinase (PI3K) pathway. For the activation of PI3K higher concentrations are necessary than for the activation of the MAPK, and only higher concentrations provide protection against MPTP toxicity. GDNF may thus have qualitatively different effects on RET-induced signalling at different concentrations.

To address these possibilities mice with a tissue-selective ablation of the gene encoding *Ret* (*DAT-Ret*$^{lx/lx}$ mice) as well as *DAT-Cre, Ret lx* control mice were used. By 2x10^8 AAV-5 GFAP-mediated GDNF overexpression in the *striatum*, the effects of high amounts of GDNF in a MPTP mouse model that is independent of RET-mediated signalling were investigated.

It was found that astrocytic GDNF expression only mediates short- and long-term protection of the nigrostriatal system from MPTP toxicity if the RET receptor is expressed. Furthermore, without the RET receptor no striatal recovery of catecholamine concentrations from MPTP toxicity could be observed.

In conclusion, there may be no alternative to an intact GDNF-RET mediated signalling in the nigrostriatal sytem, this being a prerequisite for the protective potential of GDNF and the natural regenerative capacity of the *striatum* after MPTP lesion in mice.

2 Introduction

2.1 Neurodegenerative disorders

The major causes of disability in the western society are spontaneous or traumatic lesions in the central or peripheral nervous system; the resulting disorders strongly impair quality of life in a degree rarely comparably with other diseases. Neurodegenerative diseases (NDD) are often associated with atrophy of the affected central or peripheral structures of the nervous system. Alzheimer's disease (AD), PD, multiple sclerosis (MS), Amyotrophic lateral sclerosis (ALS), prion diseases, Huntington's disease (HD) and spinocerebellar ataxias are examples of these diseases. The majority of NDDs are characterized as having multifactorial pathology, not being related to inherited mutations of specific proteins and as having advancing age as the major risk factor.

Typical features of the pathology of NDDs are e.g. oxidative stress, axonal degeneration, demyelination, protein aggregation with consequent impairment in cellular physiological function, excitotoxicity and inflammation; in many cases of NDDs the cause has not yet been identified and there exist merely symptomatic treatments rather than a cure. In order to identify the possible causative disease mechanism various experimental animal models of NDDs have been developed. Although any one animal model reflects only certain aspects of the human disease it models, animal models lead to a better understanding of specific disease mechanisms and may serve as preclinical evaluation to develop therapies in humans (Jellinger 2009; Dauer and Przedborski 2003)**.**

2.2 Parkinson's disease

Parkinson's disease is the second most common NDD after AD and affects more than 1% of 55-year-old individuals and more than 3% of those over 75 years of age (de Rijk et al., 1997). The overall age- and gender-adjusted incidence rate is 13.4 per 100,000 with a higher prevalence among males (19.0 per 100,000) than females (9.9 per 100,000) (Van den Eeden et al., 2003). PD was initially described by James Parkinson in 1817. He defined the clinical picture for the first time in his monograph "An Essay on the Shaking Palsy" (Parkinson 2002). In his letters Wilhelm von Humboldt (1767–1835) precisely described the symptoms from a patient's point of view. He interpreted the clinical signs as an accelerated aging process (Horowski et al., 1995). More than one century had to pass after his classic monograph before the central pathological feature of PD was found; the discovery of DA in the mammalian brain and the nigrostriatal dopaminergic pathway formed by the SNpc neurons (Fig. 2.1) provided the basis for the understanding that the loss of SNpc neurons leading to a striatal DA deficiency causes the major symptoms of PD (Dauer and Przedborski 2003).

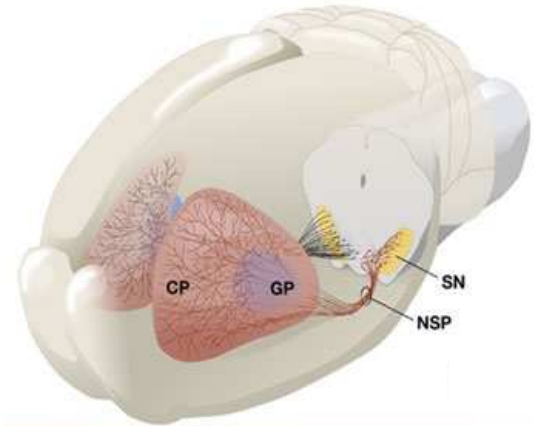

Fig. 2.1: The nigrostriatal DA pathway in the rat brain. *In the intact brain the DA producing cells reside in the SN, which is located in the ventral midbrain, and send projections (the nigrostriatal projection, NSP) to the striatum (CP), the input structure to the basal ganglia, which is situated beneath the cortex in the forebrain. The pre-terminal axons course in the medial forebrain bundle and pass through the globus pallidus (GP) before entering the target nuclei, where terminal arborizations and synaptic contacts with striatal neurons are seen. CP, caudate putamen (image taken from Kirik et al., 2004)*

2.2.1 Clinical aspects

PD or the idiopathic Parkinson syndrome is a chronically progressive NDD, in which mainly voluntary and involuntary motor skills deteriorate (motor symptoms), but also speech and other functions including thinking, mood and sensation (non-motor symptoms) (Jankovic 2008). A Parkinson syndrome is characterized by the four cardinal motor symptoms tremor, rigidity, brady-/akinesia and postural instability (Sian et al., 1999; Fahn et al., 2003).

Tremor is usually unilateral at onset, reaches its maximum during rest and decreases with voluntary movement in PD. In most cases the upper extremities are more affected than the lower ones. The term rigidity means stiffness and a increased tone of the muscles. Muscle strength is sustained; flexibility of the ankles is decreased, while the resistance increases. Movements are slowed (bradykinesia), diminished (hypokinesia) or, in severe cases completely abrogated (akinesia). An impairment of postural reflexes leads to gait and balance instability and an increased fall susceptibility (Dauer and Przedborski 2003).

Additional motor symptoms are impaired gait (decreased stride length during walking, shuffling, freezing: the inability to begin a voluntary movement such as walking, patients remain “stuck” to the ground as they attempt to begin moving), posture (camptocormia, dystonia) (Lepoutre et al., 2006), speech (hypophonia), swallowing and motor coordination performance, fatigue, reduced mimic (hypomimia) leading to a masked face (Deuschl and Goddemeier 1998) and decreased size (micrographia) and speed of handwriting. Typical

non-motor symptoms are disturbances in mood (Ishihara and Brayne 2006; Lieberman 2006), cognition (slowed reaction time “bradyphrenia”, dementia, dilution) (Frank et al., 2005), sleep (somnolence, insomnia), autonomic control (dermatitis (Gupta and Bluhm 2004), incontinence, nocturia) sensation (reduction or loss of sense of smell, dizziness, pain, impaired proprioception), affect (patients become passive and withdrawn, with lack of initiative; they may sit quietly unless encouraged to participate in activities).

Only about 75-80% of the Parkinsonian disorders corespond to PD and are referred to as primary (idiopathic) Parkinson syndromes of unknown etiology. The heredodegenerative Parkinsonism includes the rare inherited cases (~ 5%). The remaining cases are classified as symptomatic or secondary Parkinson syndromes of known etiology (including medicinally or drug abuse-induced intoxications, infarcts of the *basal ganglia*, encephalitis or traumatic brain injury, tumors, metabolic dysfunction and atypical or Parkinson-Plus syndromes as part of other neurodegenerative disorders including for example multiple system atrophy (MSA), cortico-basal ganglionic degeneration or progressive supranuclear gaze palsy.

2.2.2 Pathology

PD is the most common neurodegenerative movement disorder of the *basal ganglia*. The *basal ganglia* are a group of nuclei in the brain interconnected with the *cerebral cortex*, thalamus and brainstem that play an important role in motor control, cognition, emotions and learning. The human *basal ganglia* comprise the *striatum*, consisting of the *putamen* and *nucleus caudatus*, the *globus pallidus* (GP), which is divided into an *external* (GPe) and an *internal* (GPi) segment, the *nucleus subthalamicus* (STN), the SNpc and the *pars reticulate* (SNpr) (Blandini et al., 2000). The inhibitory output of the *basal ganglia*, via the GPi and the SNpr, is thought to be modulated by one direct pathway (striatal neurons with D1 receptors, facilitating transmission) and one indirect pathway (D2 receptors, reducing transmission) providing a positive and negative feedback in the circuit between the *basal ganglia* and the *thalamus*. Although their synaptic actions are different, the DA inputs to the two pathways lead to the same effect, namely a reduction of inhibition of the thalamococortical neurons and thus a facilitation of movements initiated in the *cortex*. In PD loss of DA input from the SNpc to the *striatum* leads to increased activity in the indirect pathway and decreased activity in the direct pathway. Both of these changes lead to increased activity in the GPi, which results in increased inhibition of thalamococortical and midbrain tegmental neurons and thus to the hypokinetic features (brady- and akinesia) of the disease (Fig. 2.2) (Kandel et al., 2000 Principles of Neural Science 4th ed.). The inhibition of nuclei in the brainstem is believed to cause gait and posture disturbances (Wichmann and DeLong 1993).

A diagnosis of PD can only be confirmed by the a *post mortem* identification of the pathological hallmarks of PD for example the loss of DA neurons in the SNpc. Approximately

60% of the SNpc DA neurons have been already lost at the onset of PD symptoms. Different modes of cell death, e.g. apoptotic, necrotic and autophagic, have been described to contribute to the neuronal loss occurring in PD (von Bohlen Und Halbach et al., 2004; Blum et al., 2001). Furthermore, the axonal projections of the SNpc dopaminergic neurons to the *striatum* are reduced, leading to depletion in DA of approximately 80% in the putamen. Thus, the degree of terminal loss in the *striatum* appears to be more pronounced than the magnitude of SNpc DA neuron loss, suggesting that striatal DA nerve terminals are the primary target of the degenerative process and that neuronal death in PD may result from a "dying back" process (Dauer and Przedborski 2003). This theory is supported by results of studies with PD animal models showing that the destruction of striatal terminals precedes that of SNpc cell bodies (Herkenham et al., 1991) and protection of striatal nerve terminals prevents the loss of SNpc DA neurons (Wu et al., 2003). If the DA content drops below a threshold of about 20%, the increase in the glutamatergic innervation of the striatal cholinergic interneurons tips the balanced ratio between DA and acetylcholine (ACh) in favour of the latter. The resultant physiological alterations in the activity of the neural circuits within the *basal ganglia* induce PD symptoms (Muller et al., 1999; Bernheimer et al., 1973).

"Lewy bodies" (LBs), another hallmark of PD are intracellular proteinaceous inclusions. LBs are spherical eosinophilic cytoplasmatic deposits with a diameter of more than 15 µm that consist of a dense granulovesicular core surrounded by a clear halo of 8-10 nm-wide radiating fibrils. Next to α-synuclein, which is their major component (Goldberg and Landsbury 2000; Spillantini et al., 1998; Spillantini et al., 1997; Pappolla 1986) the LBs contain a variety of other proteins including parkin (Shimura et al., 2001), ubiquitin (Lowe et al., 1988), ubiquitin carboxy-terminal hydrolase (UCH-L1) (Ardley et al., 2004), 2001) PINK1 (Muqit et al., 2006), cytoskeletal proteins (Schmidt et al., 1991; Ihara et al., 2003), and synphilin-1 (Wakabayashi et al., 2000).

The degenerative process of PD is not restricted to the SNpc. Mental, autonomic and endocrine dysfunctions are proposed to be caused by extranigral alterations (Braak and Del Tredici 2008). The *locus coeruleus*, the *ventral tegmental area*, the *nuclues basalis Meynert*, the *thalamus*, the *hypothalamus* and many cortical areas are other pigment-containing brain structures, which are affected to a different extent (Gibb 1997). An interesting hypothesis in this regard is the Braak theory, demonstrating that the major cellular pathology of PD, aggregate formation begins in the *dorsal glossopharyngeal-vagus complex* in the *medulla* as well as in the *anterior olfactory nucleus* and spreads up the brainstem, involving the SNc only later in its course (Braak et al., 2003; Del Tredici et al., 2002) but prior to the point that cell loss in these other structures generally reaches symptomatic thresholds; indeed olfactory dysfunction has been recognized as a very early feature of typical PD (Tissingh et al., 2001).

It is proposed that once the SNc is affected by the original pathobiological process, additional processes more specific to DA neurons are triggered (Lang 2007).

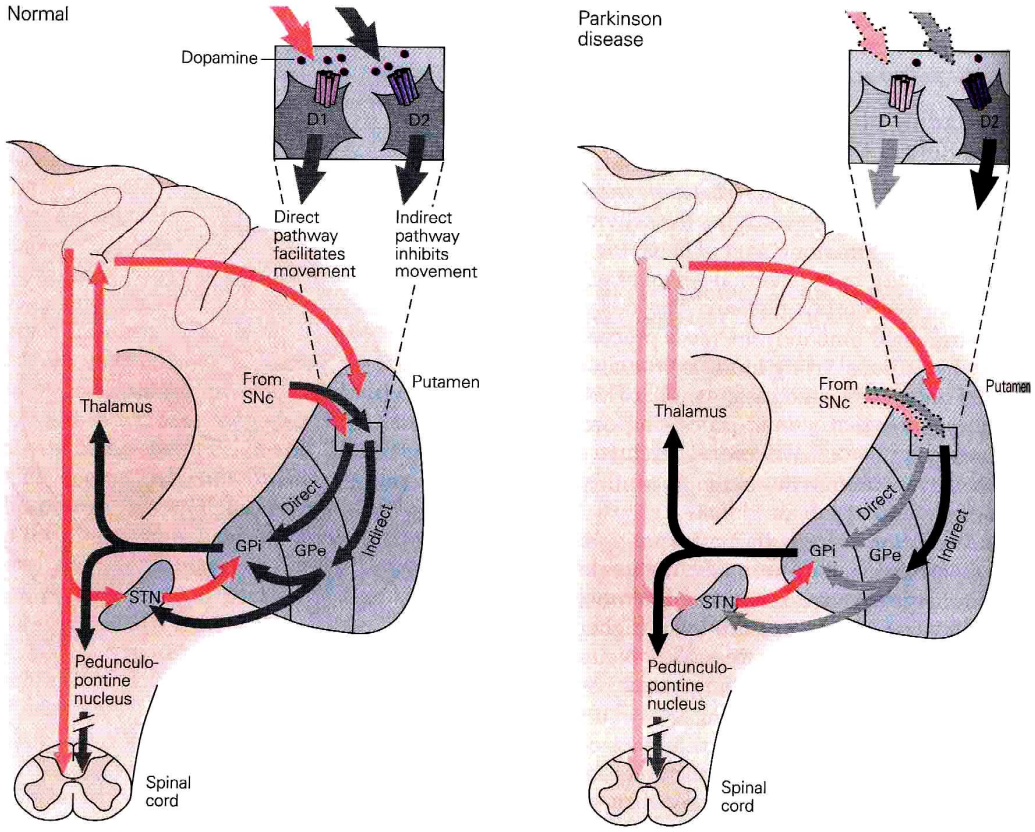

Fig. 2.2: The basal ganglia-thalamocortical circuitry under normal conditions and in PD. *Two types of DA receptors (D1 and D2) are located on different sets of output neurons in the striatum that give rise to the direct and indirect pathways. Inhibitory connections are shown as gray and black arrows; excitatory connections as pink. Degeneration of the nigrostriatal DA pathway in PD leads to differential changes in activity in the two striatopallidal projections, indicated by changes in the darkness of the connecting arrows (darker arrows indicate increased neuronal activity and lighter arrows, decreased activity). Basal ganglia output to the thalamus is increased in PD. GPe = external segment of the globus pallidus; Gpi = internal segment of the globus pallidus; SNc = substantia nigra pars compacta; STN = subthalamic nucleus (image taken from Kandel et al., 2000 Principles of Neural Science 4th ed.).*

2.2.3 PD causative hypotheses

Pathological processes, including oxidative and nitrosative stress, inflammation, mitochondrial defects, excitotoxicity, apoptosis and genetic predisposition have been implicated in the pathogenesis of PD (Moore et al., 2005; Larkin 1999). A multifactorial genesis is currently assumed, involving both genetic and environmental risk factors, since a clear toxic, environmental or genetic etiology can be identified only in the minority of PD cases (Lorincz 2006).

DA cells of the SNc are more likely to be susceptible to oxidative stress in comparison to other brain structures due to highly oxidative intracellular environment (Lotharius and Brundin 2002; Lotharius et al., 2002; Kuhn et al., 1999; Jenner and Olanow 1998; Berman and Hastings 1997). The DA metabolism leads to the formation of free radicals and other reactive oxygen species (ROS) (Aisen et al., 1990). Free radicals rapidly react with cellular macromolecules (Blandini et al. 2000) leading to a dysfunction of proteins (Bowling and Beal 1995), DNA damages (Yoritaka et al., 1996), mitochondrial deficits via dysfunction of the electron transport chain (Allen et al., 1995) and finally cell death (Halliwell 1992).

In SNc DA neurons of sporadic PD patients increased amounts of oxidative damaged lipids, proteins, DNA (Jenner 2003; Floor and Wetzel 1998) and changes in the ROS detoxification systems were found. In comparison to age-matched control subjects the enzymatic activity of catalase (CAT) and glutathione (GSH) peroxidase in pathological early PD patients are unaffected, while GSH levels are significantly decreased (Sian et al., 1994), suggesting that PD patients have a reduced H_2O_2 clearance capacity. DA is degraded to 3,4-dihydroxybenzoic acid (DOPAC) and homovanillic acid (HVA) by monoamine oxidase B (MAO-B) and catechol-O-methyltransferase (COMT) leading to the production of one molecule H_2O_2 per molecule DA. Depletion of synaptic DA during the progression of PD leads to a compensatory increased DA turnover, which might exacerbate the oxidative stress by further increasing the production of H_2O_2 (Schulz and Falkenburger 2004). A decrease of the mitochondrial complex-I activity was observed in PD (Schapira et al., 1989).

The second mechanism held to play a causative role in PD pathogenesis is protein misfolding and aggregation. Proteopathies, including PD (cytoplasmatic LBs), Alzheimer's disease (extracellular senile plaques and intracellular tau) and HD (intranuclear huntingtin inclusions) are characterized by the presence of proteinaceous deposits, built up by aggregated proteins.

The proteolytic ubiquitin-proteasome system (UPS) reduces the levels of these soluble abnormal proteins (Hanna and Finley 2007; Pickart 2001; Voges et al., 1999). Chaperones support folding, refolding or degradation of misfolded polypeptides, prevent protein aggregation and are involved in the formation of proteinaceous inclusions, called aggresomes (Opazo et al., 2008).

2.2.4 Genetic clues to the etiology of PD

Since the late 1990s genetic analyses of PD patients have been able to identify a number of loci (PARK 1-13) and genes associated with either autosomal dominant (α-synuclein, UCH-L1, LRRK2), autosomal recessive (Parkin, PINK-1, DJ-1, ATP13A2) or inherited forms of PD with incomplete penetration (synphillin-1, HtrA2/Omi) that play a causal role in PD. In support of the two PD causative hypotheses most of the proteins encoded by these genes are involved in the UPS or in mitochondrial function. Although, just a minority of PD cases (~5%) is due to genetic defects, the typical and extremely consistent phenotype of both idiopathic and familial PD suggests that they share a common molecular mechanism (Thomas and Beal 2007). Typical for familial PD is an early onset, a slowed progression of motor and cognitive symptoms and in some cases a lack of LB formation.

2.3 Therapeutic approaches for PD treatment

None of the currently available treatments have been proven to slow the progression of PD. However, medications or surgery can provide relief from the symptoms as palliative, rather than curative therapies for PD. Since the early days of experimental gene therapy for other disorders, PD was considered an ideal brain disease for this approach (Mouradian and Chase 1997), because of the relatively selective localization of the pathology, at least in the early stages of the disease, an understanding DA biosynthesis and *basal ganglia* circuitry, a knowledge of DA neurotrophic factors and the availability of animal models. Improvements in vector design have made it possible to deliver therapeutic genes directly into the brain with reasonable safety, thus having fueled the development of therapies to augment DA neurotransmission, modulate *basal ganglia* circuitry or even for neuroprotective approaches (Mochizuki et al., 2008).

2.3.1 Current therapeutical strategies and limitations

The pharmacological standard for the symptomatic treatment of PD is to substitute DA by: (i) administration of DA precursors or agonists and (ii) inhibition of DA degradation. This is typically accomplished with the dopamine precursor 1-3,4-dihydroxyphenylalanine (L-DOPA). The latter became clinical practice after being reported to improve akinesia in PD after initial studies in the 1960s (Hornykiewicz 2002; Cotzias et al., 1968). L-DOPA is administered in combination with DOPA-decarboxylase blockers such as carbidopa or benserazide, which reduce the peripheral conversion of L-DOPA to dopamine thus allowing a considerable amount of drug to cross the blood brain barrier and undergo decarboxylation to DA in the brain. As the disease progresses, up to 80% of patients experience motor fluctuations (the so-called “wearing off” and “on-off” phenomena) and dyskinesias within 5-10 years after disease onset. To delay these fluctuations DA agonists can be used as an initial and

adjunctive therapy in PD. Limits of this agonist therapy are cardiovascular and psychiatric complications (Jankovic and Stacy 2007; Lang and Lozano 1998). A further reduction of medication dosage-dependent fluctuations can be realized by novel drug delivery systems for constant administration of L-Dopa and dopamine agonists via intratestinal, transcutaneous or subcutaneous infusion (Schulz 2008).

Other medications including anticholinergic agents, inhibitors of MAO-B or COMT provide only mild to moderate benefit (Olanow and Stocchi 2004; Hristova et al., 2000; Lang et al., 1998). MAO-B inhibitors, such as rasagiline and selegiline, increase concentrations of dopamine in the brain by blocking its reuptake from the synaptic cleft. This can slow motor decline, prolong “on” time and improve symptoms of PD. Administration of COMT inhibitors in combination with L-DOPA decelerate the elimination half-life of L-DOPA, leading to decreased “off” time and increased “on” time and allow for a lower daily L-DOPA dosage, since DA and L-DOPA are metabolized by COMT. “Off” time refers to periods of the day when the medication is not working well for the patients; over the course of PD a substantial number of patients develop these fluctuations in response to medication. This process is related to the progression of PD and the reduction of a DA storage capacity.

At later stages of the disease, when symptoms are no longer adequately controllable with medications, or if medications have severe side effects, patients may benefit from neurosurgical procedures. The common principle of these neurosurgical treatments is to mimic the inhibitory function of the SNpc on its target regions, which become overactive due to the degeneration of the SNpc by preventing them from firing either via electrothermal tissue ablation or chronic, high frequency deep brain stimulation (DBS) (Kringelbach et al., 2007). The *ventrointermediate thalamic nucleus*, the GPi and the STN are the anatomic targets for that therapy.

2.3.2 Neuroprotective PD therapy

Different strategies with varying degrees of success have been employed to inhibit neurodegenerative processes; no sustained neuroprotection could be accomplished by early studies aimed at blocking the executioners of apoptotic cell death, cysteine proteases of the caspase family (Rideout and Stefanis 2001; Perrelet et al., 2000; Kermer et al., 1999). Most pro-apoptotic signals converge on breakdown of mitochondrial membrane potential, followed by release of pro-apoptotic factors and subsequent caspase activation (Chang et al., 2002). Thus several studies aimed to maintain mitochondrial integrity via the overexpression of anti-apoptotic members of the bcl-2 family of proteins (Malik et al., 2005; Wong et al., 2005; Azzouz et al., 2000). Although this approach was reported to be significantly more efficient than caspase inhibition, in long-term studies substantial neuronal cell loss was still observed (Kim et al., 2005; Malik et al., 2005). Furthermore, the MAO-B inhibitor rasagiline (Sampaio

and Ferreira 2010; Naoi and Maruyama 2009) and the complex I mitochondrial fortifier coenzyme Q10 are under clinical investigation for neuroprotective effects (Spindler et al., 2009). Neurotrophic factors in several paradigms could only shortly postpone neuronal degeneration (van Adel et al., 2003; Cheng et al., 2002). GDNF and neurturin (NRTN) appear to be an exception and remain promising candidates in the treatment of PD.

2.3.3 AAVs in PD gene therapy

Recombinant viral vectors such as lentivirus (LV), herpes simplex virus (HSV), adenovirus (AdV) and AAV have been developed to transfer genes of interest into target tissues of the central nervous system (CNS) (Mandel et al., 2008). However, since LVs integrate into the genome (Mitchell et al., 2004; Sinn et al., 2005) they are basically carrying the risk of tumor formation (Follenzi et al., 2007). Furthermore, in comparison to LVs, AAV vectors seem to be more efficient in transducing the brain, thereby requiring less vector to transduce a unit area (Manfredsson et al., 2009). In view of neuroprotective applications the advantage of AdVs being retrogradely transported to the SNpc following injection into the *striatum* (Peltekian et al., 2002; Soudais et al., 2000) also has to be relativised by safety concerns, because of immune responses to AdV infection (Lowenstein et al., 2007; Puntel et al. 2006; Muruve et al., 2004; Lowenstein and Castro 2003).

Wild-type (wt) AAV is not known to be associated with any disease in humans or mammals and shows no inherent vector toxicity, which makes it an attractive tool for human gene therapy. A short viral production time, long-lasting transgene expression from the recombinant genome and in combination with cell specific promoters an expression exclusively in target cells are further benefits of AAV vector systems (Shevtsova et al., 2005; Kügler et al., 2003; Peel and Klein, 2000).

To date, 10 different AAV serotypes have been identified (AAV1-9 and AAVRh10) of which AAV1-, AAV2-, AAV5-, AAV8- and AAV10-derived viral vectors have been used in the CNS (Mandel et al., 2008). Studies have demonstrated that AAV can infect lower motor neurons after injection into the sciatic nerve, followed by retrograde transport and transgene expression (Kaspar et al., 2003; Pirozzi et al., 2006). A comparison of the retrograde transport efficiency between serotypes revealed AAV-1 with greatest efficiency of retrograde transport after intramuscular injection. Serotypes 2-6 showed a low efficiency for retrograde transport (Hollis et al., 2008). The various serotypes differ in structure and bind unique membrane-associated receptors, resulting in differences in cell tropism and virus spread among the serotypes. The most extensively studied serotypes are AAV-2, the only AAV serotype in active clinical trials, and AAV-5 (Hildinger and Auricchio 2004).

Recombinant AAV vectors are considered to have one of the highest biosafety ranking among the viral vectors (Mandel et al., 2008). Furthermore, deletion of the wt rep gene

impairs their propensity for the site-specific integration in the chromosomal DNA ending up mainly in an episomal form of the AAV DNA (Duan et al., 1998). Nevertheless, rAAV have been proven to mediate stable transgene expression for more than one year (Stieger et al., 2006; Woo et al., 2005). Furthermore, AAVs are capable of infecting both dividing and non-dividing cells (Flotte et al., 1994; Flotte et al., 1992).

In a phase 1 trial of AAV-2-mediated expression of glutamic acid decarboxylase (GAD) in the STN, neither antibody responses to either the AAV vector or the expressed transgene occurred during the first 12 months, demonstrating that AAV gene therapy is safe and well tolerated by patients with advanced PD. Since GAD catalyses the synthesis of γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain and since in PD patients activity of the STN is increased mainly because of reduced GABAergic input from the GP, the GAD expression led to a balance of this increased STN activity in PD patients (Kaplitt et al., 2007). Additionally, another phase I clinical safety trial using bilateral intraputaminal infusion of an AAV-2 expressing the human aromatic l-amino acid decarboxylase (hAADC), which catalyses the conversion of L-DOPA to DA also declared AAV gene therapy as safe. In this study they detected after (non-blinded) analysis of a first small cohort a good tolerance of the viral vector infusion and (in the absence of controls) revealed an average 30% increase in striatal 6-[^{18}F]-fluoro-L-dopa uptake capacity as a marker for DA synthesis in a low-dose group and 75% in a high-dose group (five patients each). Total and motor rating scales improved in both cohorts and motor diaries revealed increased on-time and reduced off-time dyskinesia (Christine et al., 2009). Preliminary, open label evidence in PD subjects offered support for CERE-120 (AAV-NRTN) as a novel PD therapy, possibly restoring function of degenerating DA neurons and preventing further degeneration (Marks et al., 2008; Bartus et al., 2007). However, latest results of a double-blind phase 2 clinical trial showed no convincing clinical benefit (Bartus 2009, Scientific Symposium 113: Late stage industry clinical trials).

2.4 Animal models of PD

Modelling human neurological disorders in animals is common practice used for the study of underlying pathogenetic mechanisms and evaluation of novel therapeutic approaches. Various animal models currently in use can be subdivided into two groups: 1) genetic and 2) toxic models. The first group continuously enlarges due to the discovery of new mutations in PD-related genes as well as due to successful transgenic technologies, the use of different model systems (ranging from Drosophila, via mice to primates) and gene transfer approaches. The toxic models of PD remain a widely employed alternative, since they are easy to create and are able to reproduce the major etiopathologic features of PD induced by oxidative stress and inhibition of complex I. Several different neurotoxins, 6-

hydroxydopamine (6-OHDA), MPTP, paraquat and rotenone are commonly used to this purpose (von Bohlen and Halbach et al., 2004; Blum et al., 2001).

Since 6-OHDA cannot cross the blood brain barrier, it requires direct application into the brain. Its preferential uptake by DA and noradrenaline transporters causes the specific toxicity for catecholaminergic neurons after cytosolic accumulation and formation of ROS and toxic quinones (Terzioglu and Galter 2008; Bove et al., 2005). Moreover, 6-OHDA has been demonstrated to impair enzyme activity of cellular anti-oxidants like GSH and SOD, which can further enhance destructive effects of oxidative stress (Schober 2004).

The herbizide paraquat has structural similarities to MPTP, but is not selectively taken-up by DAT and thus does not accumulate in DA neurons after systemic administration. It induces a modest, specific loss of DA neurons in the SNpc (Ossowska et al., 2006; McCormack et al., 2002) by redox cycling with cellular diaphorase such as nitric oxide synthase producing ROS.

Rotenone is produced in the roots and stems of tropical leguminosa plants, having been widely used around the world as insecticide, pesticide and piscicide. By inhibiting the transfer of electrons from complex I to ubiquinone in the mitochondrial electron transfer chain, it affects mitochondrial function at the same site as 1-Methyl-4-phenylpyridinium $(MPP)^+$, but is only mildly toxic for humans.

2.4.1 MPTP

So far, none of the validated toxic models faithfully reproduce PD. However, among these, the MPTP model has several advantages over the others. MPTP induces a syndrome in humans and monkeys almost indistinguishable from PD with an irreversible lesion of the nigrostriatal DA pathway and even though safety measures are important for its use neither surgery nor particular equipment is required (Jackson-Lewis and Przedborski 2007; Langston et al., 1986), thus making it ideal to study PD in animal models. Responses in the MPTP animal model, as well as the complications to traditional anti-Parkinsonian therapies are virtually identical to those seen in PD (Dauer and Przedborski 2003).

MPTP was recognized in 1982, when young drug addicts developed an irreversible, rapidly progressing Parkinsonian syndrome following the intravenous injection of an illicit preparation of 1-methyl-4-phenyl-4-propionpiperidine (MPPP), an analog of the narcotic meperidine (Demerol), contaminated with MPTP, which was inadvertently synthesized as a by-product of MPPP (Langston et al., 1983).

From neuropathological data, we know that MPTP administration causes -as seen in PD-, the disruption of mitochondrial oxidative phosphorylation, oxidative stress, degeneration of DA neurons in the SNpc and depletion of striatal DA levels (Forno et al., 1993). Additionally, an increased expression of inflammatory markers colocalizing with microglia has been observed (Liberatore et al., 1999; Hunot et al., 1999). On the other hand, two typical

neuropathological features of PD have, until now, been lacking in the MPTP model. First, neurons are not consistently lost from other monaminergic nuclei, such as the *locus coeruleus*. Secondly, the eosinophilic intraneuronal inclusions called Lewy bodies, characteristic of PD, have not thus far been convincingly observed in MPTP-induced Parkinsonism (Forno et al., 1993). However, continuous MPTP infusion by osmotic minipumps triggered formation of ubiquitin and α-synuclein positive nigral inclusions in mice (Fornai et al., 2005). Due to its lipophilic character, MPTP can easily pass cell membranes and the blood brain barrier. In the brain, specifically in astrocytes, it is converted into the active toxic metabolite MPP^+ by the enzyme MAO-B. MPP+ is selectively taken up into DA neurons by DAT and after transportation into mitochondria it inhibits complex I of the mitochondrial respiratory chain. This promotes ATP depletion and generation of ROS, finally activating apoptotic pathways (Przedborski et al., 2004; Ghahremani et al., 2002; Lei et al., 2002). Inflammation, the activation of excitatory amino acid receptors, apoptosis, and autophagia as cell death mechanisms have been implicated in MPTP toxicity (Dawson 2000; Beal 2001). The susceptibility to MPTP seems to depend on MAO-B activity and on the capacity to sequester MPP^+ into synaptic vesicles via the vesicular monoamine transporter (VMAT) as cellular protective mechanism, probably based on the ratio of DAT and VMAT (Miller et al., 1999; Takahashi et al., 1997). This can be an explanation for mouse strains reacting very differently to the toxin, whereas humans are sensitive to MPTP intoxication at quite low doses. The impact of systemic MPTP treatment in mice depends on the regimen of administration and induces either loss of DA-ergic neurons in the SNpc and striatal DA depletion alone, or in addition, motor symptoms including bradykinesia, rigidity and posture abnormalities (Sedelis et al., 2000). If neurons are estimated shortly after MPTP treatment the TH immunoreactivity loss may not reflect the actual DA cell death, since MPP^+ down-regulates TH gene expression (Xu et al., 2005).

2.5 Potential of growth factors for PD treatment

One treatment area that has gained significant momentum over the last several years has been the use of various growth factors aimed at halting or slowing the progressive loss of striatal DA innervation. Several candidate molecules have been proposed including GDNF, NRTN, brain-derived neurotrophic factor (BDNF), conserved DA neurotrophic factor (CDNF), mesencephalic astrocyte-derived neurotrophic factor (MANF), insuline-like growth factor (IGF) and basic fibroblast growth factor (bFGF). All these candidates offer protective potential but due to strong side effects (BDNF) or the lack of sufficient preclinical data (CDNF, MANF, IGF, bFGF) to date only GDNF and its relative NRTN have progressed to PD clinical trials (reviewed by Manfredsson et al., 2009).

2.5.1 Neurotrophic factors

Endogenous neurotrophic factors regulate physiological cell death during neuronal development, facilitate target innervation, neurite branching and synaptogenesis, maintain the survival of neurons during postnatal life and regulate adult synaptic plasticity and maturation of electrophysiological properties. The neurotrophic theory postulates the function of neurotrophic factors primarily through a retrograde mechanism with the basic tenants of 1) limited quantities of a neurotrophic factor are produced in a specific target tissue; 2) responsive neurons projecting to these targets compete for this limited quantity of trophic factor and die if unable to access adequate amounts; 3) the factor is then bound to cell surface receptors; 4) receptors and trophic factor are internalized; and 5) retrogradly transported to the neuronal cell nucleus (Peterson and Nutt, 2008). Neurotrophic factors include neurotrophins (for example nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3)), neurokines and GDNF family ligands (GFLs). In addition to GDNF, artemin (ARTN), NRTN and persephin (PSPN) belong to the group of GFLs distantly related to the transforming growth factor-beta superfamily, containing seven cystein residues with the same relative spacing and acquiring similar conformation as the other members of this superfamily (Airaksinen and Saarma, 2002, Ibanez 1998). Possible actions by which trophic factors could provide important therapeutic effects in PD are 1) the promotion of DA SNpc neuron survival; 2) the restoration of neuronal function regarding their DA phenotype (the capacity to synthesize and release DA); and 3) the stimulation of the DA system since acute effects on ion channels causing changes in cell excitability occur with some trophic agents. Finally, it is possible that loss or disruption of specific trophic factors, their receptors or their signal cascades cause PD (Peterson and Nutt 2008).

2.5.2 GDNF

Although GDNF was originally purified from rat glioma cell line supernatant and thus termed glial cell line-derived derived (Lin et al., 1993), striatal GDNF expression in the developing brain is largely neuronal, as astroglial cells were not found to express detectable GDNF (Oo et al., 2005). However, upon injury, glial cells appear to become the predominant source of GDNF (Bresjanac and Antauer 2000; Nakagawa and Schwartz 2004; Chen et al., 2006). Astrocytes are key elements in the brain response to injury since upon activation they up-regulate antioxidant molecules, membrane transporters, and trophic factors that support neuronal and glial survival and tissue repair (reviewed by Liberto et al., 2004).

GDNF was first characterized as a trophic factor that supports differentiation and survival of midbrain dopaminergic neurons (Lin et al., 1993) but GDNF also supports motor neurons (Henderson et al., 1994), noradrenergic neurons (Arenas et al., 1995), sensory and autonomic neurons (Trupp et al., 1995). In the developing and adult rat nervous system,

GDNF is present in the thalamus, hippocampus, cerebellum, cortex, *striatum* and spinal cord (Trupp et al., 1997; Pochon et al., 1997; Choi-Lundberg and Bohn 1995; Stromberg et al., 1993). In the developing rat nervous system GDNF levels in the SN are much lower compared to the *striatum* (Oo et al., 2005, Choi-Lundberg and Bohn 1995) suggesting a role for GDNF mainly as a target derived neurotrophic factor for DA neurons. This part of the neurotrophic theory for GDNF gets further support by the fact that GDNF is transported retrogradely from the *striatum* to the DA cell bodies in the SNpc and that there is no GDNF mRNA but rather only GDNF protein detectable in the SNpc (Barroso-Chinea et al., 2005; Oo et al., 2005; Choi-Lundberg and Bohn 1995; Tomac et al., 1995).

GDNF is a glycosylated, disulfide-bonded homodimer, with a molecular weight of 33-45 kDa, while the monomer has a molecular weight of 16kDa after deglycosylation (Lin et al., 1994). Sequence data suggest that GDNF is synthesized as an active 211 amino acids/long pre-proGDNF, and then processed to the mature protein with 134 amino acids (Lin et al., 1993). GDNF expression can be positively regulated by neurotransmitters (for example DA, serotonin, glutamate), pro-inflammatory molecules (for example lipopolysaccharide (LPS), interleukine (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and TNF-β), hormones (for example melatonin, vitamin D_3, Oestrogen), signalling pathways (for example mitogen-activated protein kinase (MAPK), protein kinase C (PKC), Ca^{2+}) and transcription factors (for example NF-κB, cAMP response element-binding protein (CREB)); a negative regulation has been revealed for molecules reducing oxidative stress (NF-κB inactivators), IL-10 and type-2A protein phosphatases (PP) (Saavedra et al., 2008).

Since the levels of other growth factors are decreased in PD brains, it was suggested that the unchanged levels of GDNF in PD might be due to compensatory production by glia cells (Mogi et al., 2001). However, large reductions in GDNF content are reported in surviving PD SN neurons (Chauhan et al., 2001) and increased levels of a GDNF isoform were found in the putamen of PD patients with marked neuronal loss (Backman et al., 2006). Interestingly, GDNF up-regulation can also be detected in the SN and *striatum* after MPTP intoxication. Whatever might be the endogenous changes of neurotrophic factors in PD, a therapy with GDNF might prevent the progression of the disease and restore function (Slevin et al., 2005; Gill et al., 2003).

2.5.3 GDNF signalling

Until recently, neither GDNF nor its receptors have been utilized in gene ablation analyses to study the effects of GDNF on DA neurons in vivo because all of the engineered mice were neonatally lethal (for review, (Baloh et al., 2000)). GDNF signals through a two-component receptor complex consisting of the RET receptor tyrosine kinase, which was initially discovered as protooncogene (Takahashi 2001) and the GPI-linked GDNF family receptor

alpha (GFRα)1. To date four members of this family have been identified (GFRα1-4). GFRα1 and RET have been detected via Northern blot, PCR, and in situ hybridization analysis in DA neurons from the SN (Nosrat et al., 1997; Trupp et al., 1997, 1996; Treanor et al., 1996). In addition to the known receptors GFRα1 and RET, GDNF signalling and the cellular response to GDNF requires heparan sulphate glycosaminoglycans (Barnett et al., 2002; Tanaka et al., 2002). RET is activated upon binding of a GDNF dimer to GFRα1 linked to the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor. Dimerization of RET triggers its autophosphorylation, thus initiating several downstream signalling molecules, prominently extracellular signal-regulated kinase (ERK)1/2 (Kaplan and Miller 2000), that regulate cell survival, proliferation, differentiation, neurite outgrowth, synaptic plasticity and morphogenesis (Airakasinen and Saarma 2002) (Fig. 2.3 A). However, the ability of GDNF to protect DA neurons cannot be explained solely in terms of its influence on ERK1/2, since one study revealed maximal increase in ERK1/2 phosphorylation already at 0.45µg but GDNF started its protective activity at a concentration of 4.5µg (Lindgren et al., 2008). Phosphatidylinositol 3-kinase (PI3K)/Akt signalling maintains viability through antiapoptotic effects and it mediates effects on axonal caliber, branching and regeneration (Brunet et al., 2001; Markus et al., 2002; Namikawa et al., 2000). In neurons, Akt activation has been identified in response to treatment with IGF-1 (Dudek et al., 1997), NGF (Soltoff et al., 1992) and GDNF (Creedon et al., 1997). AAV Myr-Akt (constitutively active form) pronounces trophic effects on DA neurons of adult and aged mice and the transduction in the SNpc conferred almost complete protection against apoptotic cell death in the 6-OHDA Parkinson model (Ries et al., 2006). Thus the PIK3/Akt signalling pathway could be associated with the neuroprotective effects of GDNF (Lindgren et al., 2008).

The Met918Thr mutation leads to constitutive activity of RET, causing the cancer syndrome called multiple endocrine neoplasia type B (MEN2B). Constitutive RET activity in knock-in MEN2B mice induces a profound elevation of brain DA concentration via enhanced synthesis and increases the number of TH-positive cells in the SN (Mijatovic et al., 2007). However, two recent studies surprisingly demonstrated that conditional ablation of RET using Cre recombinase under control of the dopamine transporter (DAT)-Cre did not disturb the development of the nigrostriatal dopaminergic pathway (Jain et al., 2006; Kramer et al., 2007). While in one study there was no degeneration of the nigrostriatal pathway up to 12 months of age (Jain et al., 2006) the other study reported a loss of TH-positive neurons in the SNpc and TH-positive terminals in the *striatum* starting at 12 months and progressing thereafter (Kramer et al., 2007). Tissue selective ablation of the RET receptor in DA neurons does not modulate the MPTP-induced degeneration of SNpc DA neurons and their terminals in the *striatum* in 3-4 months old mice but RET is required for the regeneration of DA axon terminals. Thus GDNF/RET signalling has an important impact on the regenerative capacity

of the nigrostriatal system (Kowsky et al., 2007). In most studies examining its neuroprotective potential, high concentrations of GDNF (10µg or more) were used (Salvatore et al., 2004; Winkler et al., 1996; Lapchak 1996; Kearns and Gash et al., 1995; Tomac et al., 1995; Hoffer et al., 1994). This seems inconsistent with *in vitro* experiments showing high-affinity binding of GDNF to GFRα1/RET to be in the nanomolar to picomolar range (Trupp et al., 1998; Sanicola et al., 1997; Jing et al., 1996; Treanor et al., 1996).

GDNF may also signal indepently of RET but involve the same receptor GFRα1. In RET-deficient cells GDNF may act through the Src family kinases, inducing sustained activation of the Ras/ERK and PI3K/Akt pathways (Tupp et al., 1999). In the absence of RET, Src-mediated cellular events may also promote survival and neurite outgrowth (Sariola and Saarma 2003). NCAM has been proposed as an alternative signalling receptor for GDNF (Paratcha et al., 2003). In the absence of GFRα1, GFL associates with NCAM with low affinity. In turn in the presence of GFRα1, GDNF may bind to the p140-NCAM and activate cytoplasmic Src-like Fyn and FAK kinases (Paratcha et al., 2003) (Fig. 1.3 B). Interestingly, in rats both *in vivo* and *in vitro* effects of GDNF, such as DA neuron survival, neurite outgrowth, DA turnover and locomotor activity were inhibited by anti-NCAM antibodies (Chao et al., 2003). Also interesting is an *in vitro* study revealing that neurons express GFRα1 and RET, whereas astrocytes express not only but predominantly GFRα1, suggesting that they might utilize separate pathways to mediate autocrine and paracrine effects of GDNF (Sandhu et al., 2009).

Other findings suggest that integrin β1, another adhesion molecule, is also involved in GDNF signalling (Cao et al., 2008). Negatively acting players on GDNF signalling are for example the transmembrane protein Lrig1 and Gas1, which show high structural similarity to GFRα (Ledda et al., 2008; Lopez-Ramirez et al., 2008; Cabrera et al., 2006; Schueler-Furman et al., 2006).

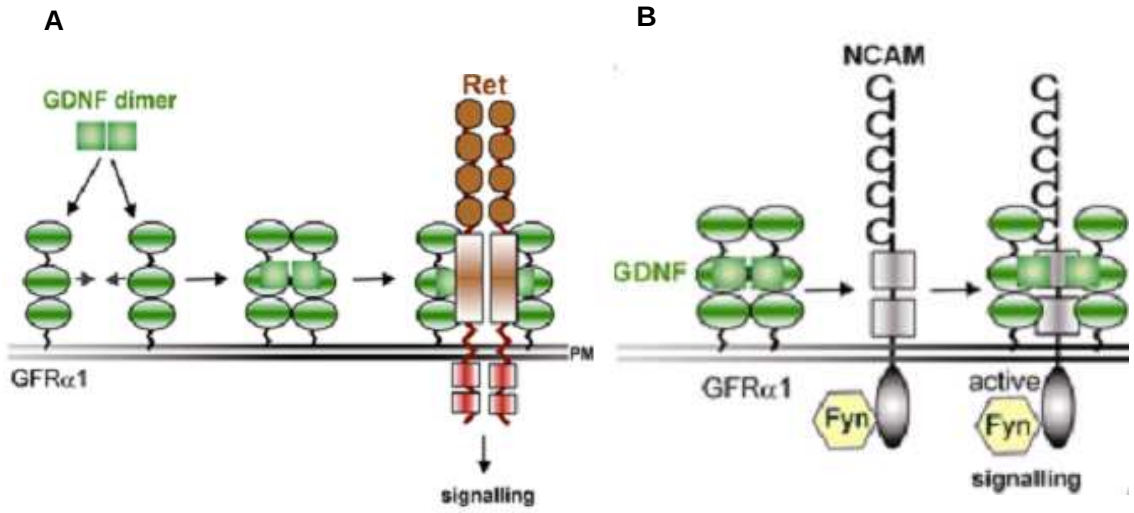

Fig. 2.3: GDNF receptor interaction. *(A) A dimer of GDNF brings together two molecules of GFRα1. This complex dimerizes two molecules of RET leading to transphosphorylation of their tyrosine domains. (B) NCAM interacts with a GDNF-GFRα1 dimer leading to activation of Fyn, a SRC-like kinase (image taken from Sariola and Saarma 2003).*

2.5.4 GDNF in PD Models

In culture, GDNF is a survival factor for primary mesencephalic DA neurons and protects them against a number of toxic insults (Krieglstein 2004). *In vivo*, the protective effects of GDNF against 6-OHDA-induced loss of DA nigrostriatal neurons are undisputed (Lindgren et al., 2008; Smith and Cass 2007; Shevtsova et al., 2006; Eslamboli et al., 2005; Bilang-Bleuel et al., 1997; Mandel 1997 et al., 1997; Choi-Lundberg et al., 1997). In addition, many (Elsworth et al., 2008; Chen et al., 2008; Luan et al., 2008; Schober et al., 2007; Gao et al., 2003; Palfi et al., 2002; Costa et al., 2001; Kordower et al., 2000**;** Date et al., 1998**;** Zhang et al., 1997; Kojima et al., 1997**;** Gash et al., 1996; Tomac et al., 1995) but not all reports (Dietz et al., 2006; Eberhardt et al., 2000) using either virus-mediated GDNF expression or direct intraparenchymal GDNF delivery showed protective and regenerative effects against MPTP toxicity in animal models (Tab 2.1).

Importantly, the effect of GDNF is dependent on the route of delivery, because GDNF released into the SN is unable to protect axons and axon terminals in the *striatum* against striatal 6-OHDA but is highly efficient in protecting nigral cell bodies (Winkler et al., 1996). However, GDNF applied to the *striatum* preserves the entire nigrostriatal pathway and the animals retain their performance in motor tests (Kirik et al., 2004 and 2000).

Furthermore, the type of cells transduced plays a role and astrocytes, which perform many functions critical for neuronal survival, are very promising canditates for GDNF delivery

(Sandhu et al., 2009; Jakobsson et al., 2004; Byrd et al., 2004; Dringen and Hirrlinger 2003; Wilson 1997). Interestingly, regarding astroglial expression levels of GDNF in the murine *striatum* using the GFAP promoter (LV-GFAP GDNF) a threefold increase can be detected after intrastriatal lesion (Jakobsson et al., 2004). Up-regulation of GFAP is well documented in several animal models and pathological states in the human brain (Eng et al., 2000). The regulation of a transgene with a GFAP promoter in a viral vector give rise to high transgenic expression in the lesioned brain and a less pronounced in the intact brain, thus providing an option of regulating expression by using the machinery of the host cell instead of depending on recombinant regulatory proteins (Jakobsson et al., 2004). Furthermore, *in vitro* overexpression and long term secretion of GDNF does not alter astrocytic morphology or their proliferation rate; although astrocytes transduced with a cytomegalovirus (CMV) promoter secrete ~ 10-fold higher concentrations of GDNF in comparison to cells transduced with the GFAP promoter (already 35-fold increase to background level), a concomitant increase in neuroprotection against 6-OHDA cannot be observed (Sandhu et al., 2009). This indicates that higher amounts of GDNF are not always beneficial and might account for side effects (Arvidsson et al., 2003**;** Gill et al., 2003; Zurn et al., 2001; Kordower et al., 1999). However, there also seems to exist a threshold under which GDNF is also no longer protective (Costa et al., 2001, Zhang et al., 1997). This concentration-dependent effect of GDNF can be partly explained by opposite effects of low and high striatal GDNF levels on the TH expression in DA neurons (Georgievska et al., 2002 a and b; Kordower et al., 2000).

In conclusion, the effect of GDNF is dependent on protein levels, degree and pattern of receptor expression on cells in the damaged area, route of GDNF delivery, type of cells transduced and GDNF actions on neuronal transmission at the site of delivery (Kirik et al., 2004; Arvidsson et al., 2003).

Table 2.1: GDNF in PD models. *The table represents a selection of references.*

Model system	GDNF application	Effect	Reference
6-OHDA, cell culture	***460pg GDNF/ml LV-CMV GDNF; 36pg GDNF/ml LV-GFAP GDNF***	***neuroprotection***	*Sandhu et al., 2009*
6-OHDA, mice	***4.5µg hr GDNF, striatum***	***protection of striatal DA terminals***	*Lindgren et al., 2008*
6-OHDA, rat	***5µg hr GDNF, striatum***	***reduced oxidative stress and protection against loss of striatal and nigral DA***	*Smith and Cass 2007*
6-OHDA, rat	***AAV-2 hSyn1 GDNF, SN***	***neuroprotection***	*Shevtsova et al., 2006*
6-OHDA, primates	***AAV-2 chicken β-actin (CBA) GDNF ~40pg/mg, striatum***	***prevention of nigrostriatal degeneration, loss of striatal DA and motor dysfunction***	*Eslamboli et al., 2005*
6-OHDA, rat	***25µg GDNF, SN; 25µg GDNF, striatum***	***neuroprotection but deleterious effects on striatal fibre density and motor function; prevention of nigrostriatal degeneration and motor dysfunction***	*Kirik et al., 2000*
6-OHDA, rat	***AdV-Rous Sarcoma Virus (RSV) GDNF, striatum***	***protection of SN DA neurons and motor function***	*Bilang-Bleuel et al., 1997*
6-OHDA, rat	***AAV-CMV GDNF, SN; AdV- GDNF, SN; 10µg GDNF/day, SN***	***neuroprotection***	*Mandel et al., 1997; Choi-Lundberg et al., 1997; Winkler et al., 1996*
MPTP, primates	***AAV–2 GDNF, striatum***	***protection of implanted fetal DA neurons***	*Elsworth et al., 2008*
MPTP, mice	***AAV – GDNF, striatum***	***prevention of striatal loss of DA***	*Chen et al., 2008*
MPTP, primates	***22.5µg GDNF/day, putamen***	***preservation of motor function***	*Luan et al., 2008*
MPTP, mice	***5µg hr GDNF, striatum***	***prevention of nigrostriatal degeneration and loss of striatal DA***	*Schober et al., 2007*
MPTP, mice	***Tat-GDNF***	***no neuroprotection***	*Dietz et al., 2006*
MPTP, mice	***AdV-GDNF***	***prevention of loss of striatal DA***	*Gao et al., 2003; Kojima et al., 1997*
MPTP, primates	***LV-GDNF, caudate nucleus, putamen, SN***	***increase in striatal DA neurons***	*Palfi et al., 2002*
MPTP, mice	***AdV-CMV GDNF ~ 1ng/ml, striatum***	***no neuroprotection, preservation of loss of striatal DA***	*Eberhardt et al., 2000*

MPTP, primates	***starting from 100-500µg GDNF 9 and 13 weeks post MPTP, intraventricular; starting from 100-1000µg GDNF/4 weeks, intracerebroventricular***	***preservation of motor function***	*Costa et al., 2001 Zhang et al., 1997*
MPTP, primates	***LV-GDNF ~ 3.5ng/mg, putamen***	***prevention of nigrostriatal degeneration and motor dysfunction***	*Kordower et al., 2000*
MPTP, mice	***10µg GDNF, striatum***	***striatal recovery***	*Date et al., 1998*
MPTP, primates	***150µg SN, 450µg intracerebral/4 weeks***	***prevention of nigrostriatal degeneration***	*Gash et al., 1996*
MPTP, mice	***10µg hr GDNF, striatum and SN***	***prevention of nigrostriatal degeneration and motor dysfunction***	*Tomac et al., 1995*

2.5.5 GDNF in clinical trials

Because of its potential to maintain function and survival of DA neurons, GDNF application has been evaluated for its effects in several clinical trials. In a first trial intracerebroventricular injections of GDNF in humans did not result in any symptomatic benefit or slowing of disease progression, likely because of limited intraparenchymal diffusion of GDNF (Kordower et al., 1999 (500-4000µg rh GDNF/month)). Unilateral or bilateral putamenal infusions led to substantial beneficial effects in three phase I open-label safety trials (Slevin et al., 2007 (30 µg recombinant human (rh) GDNF per day) and 2005 (3-30µg rh GDNF/day); Gill et al., 2003 (14.4µg rh GDNF/day)). However, a similar paradigm but using an intraputaminal catheter with a slightly larger outside diameter was not successful in 34 Parkinson's disease patients in a double-blinded placebo controlled study (Lang et al., 2006 (15µg rh GDNF/day)). A major question relates to the distribution of GDNF in the putamen by the different infusion techniques. Not only methodological questions remain to be resolved (Sherer et al., 2006), but also the molecular and signalling effects of GDNF to better characterize and dissect its protective and/or regenerative potential for DA neurons. The uncertainties surrounding the trials of intraputamenal infusion of GDNF, in which no toxicity was attributable to GDNF, should not end the research for therapeutic benefit from neurotrophic factors (Peterson and Nutt 2008), but some of the investigators involved the clinical studies have recently stated that gene transfer would probable be a potentially better alternative delivery method for GDNF (Peterson and Nutt 2008).

3 Objectives

3.1 Astrocytic versus neuronal expression of GDNF: implications for gene therapy

Since GDNF has been identified in preclinical experiments as an important differentiation and survival factor for DA neurons of the midbrain but clinical trials have shown inconsistent efficacy for this study it was proposed to study and better characterize the effects of GDNF in an *in vivo* model of PD.

In this study it will be analyzed if cell-specific GDNF expression, directed to either selective astrocytic or neuronal GDNF production, delivers a safe and efficient supply of the nigrostriatal system with GDNF in PD-like experimental paradigms. It is hypothesized that GDNF produced by astrocytes might be as effective as neuronal GDNF, since, although neurons being the physiological cellular basis of striatal GDNF expression during development of the brain, astrocytes become the predominant source of GDNF in the adult brain upon injury. Furthermore, it is thought that GDNF production by astrocytes might circumvent side-effects, seen in clinical trials. This assumption is based on the fact that vector-mediated GDNF overexpression in the brain, once neurons are transduced, leads to the release of GDNF in all terminal projection areas of that neuron with uncontrollable effects. In contrast, astrocytic production of GDNF would be limited to the target area. As vector-mediated GDNF gene therapy in PD patients would last the rest of a patient's life this safety issue is of great importance.

In a first step the requirement for a system allowing to distinguish between production of GDNF from astrocytes and neurons by using cell specific viral constructs will be realized. Since gene therapy vectors based on recombinant AAVs have become widely accepted for transduction of genes into the brain, showing no inherent vector toxicity in combination with a short viral production time and long-lasting transgene expression, it was decided in favour of AAVs. Furthermore, AAV-5 expressing a transgene under the control of the GFAP or SYN promoter guarantees an expression exclusively in astrocytes or neurons with a broad spatial distribution. One of the major problems of the inconsistent outcome of GDNF in clinical trials was suggested as being related to the insufficient distribution of GDNF in the putamen by different infusion techniques.

In a next step a model has to be established in which virus-mediated production of GDNF leads to a robust protection against a nigrostriatal lesion. In this connection, it was decided in favour of the subchronic MPTP model in mice, given that MPTP produces an irreversible and severe Parkinsonian syndrome that replicates many PD features. Furthermore, regarding the question about the site of GDNF administration (SN versus *striatum*), it is choosen the *striatum*. The benefit of GDNF delivery to the *striatum* is widely accepted; in contrast, functional restoration after SN administration is discussed controversially.

After establishing a model using high viral titres in a first step (described above), it is planned to also test low viral titres in order to find the safest and most effective conditions, while still receiving protective effects of GDNF in the model.

This study will provide information derived from the subchronic MPTP mouse model about (i) optimized safety and efficacy for GDNF delivery to damaged DA neurons of the nigrostriatal system and (ii) protective and regenerative effects of GDNF to that system, thus partaking in the achievement of the goal to use neurotrophic factors in PD gene therapy.

Questions:

1) Is it possible to express GFP and GDNF specifically in astrocytes and neurons in the murine *striatum* by using $2x10^8$ AAV-5 GFAP GFP, AAV-5 GFAP GDNF and AAV-5 SYN GFP, AAV-5 SYN GDNF? Does a reduction of the viral titre to $2x10^7$ transducing units lead to any changes regarding expression level, distribution or cell specifity?
2) How much GDNF can be produced in the ipsilateral and contralateral *striatum* and SN by injecting $2x10^8$ and $2x10^7$ AAV-5 GFAP GDNF and AAV-5 SYN GDNF? Can we detect differences depending on the cellular source of GDNF?
3) Does the injection of $2x10^8$ AAV-5 expressing a transgene lead to inflammatory tissue reactions? Would such a reaction be dependent on the cellular source of transgene expression or would it change over time? Does a reduction of the viral titre to $2x10^7$ transducing units ameliorate the situation?
4) Is it possible to detect any GDNF-mediated protective or regenerative effects in the nirgo-striatal system after MPTP intoxication and unilateral, striatal stereotaxic injection of $2x10^8$ AAV-5 GFAP GDNF and AAV-5 syn GDNF? Can even after a reduction of the viral titre to $2x10^7$ transducing units the same effect be achieved?

3.2 RET signalling: the basic requirement for GDNF-mediated protection against MPTP toxicity

GDNF classically signals through a two-component receptor complex consisting of RET and GFRα. It has already been observed in this laboratory that endogenous GDNF-mediated RET signalling has no influence on the survival of DA neurons in the MPTP model of PD, but rather facilitates the regeneration of DA axon terminals in the *striatum* in longitudinal studies. Following these results endogenous GDNF does not provide protective but only restorative effects by inducing a sprouting response. The predictive value of these previous results is limited by their dependence on physiological GDNF concentrations, whereas under treatment conditions several times higher concentrations may be achieved. Furthermore, it has been claimed that GDNF at higher concentrations may also signal through others than the classical RET and GFRα receptors. To address these possibilities mice with a tissue-selective ablation of the gene encoding *Ret* (*DAT-Ret$^{lx/lx}$* mice) as well as *DAT-Cre, Ret lx* control mice and AAV-mediated GDNF overexpression in the *striatum* will be used, thus investigating the effects of GDNF in a MPTP mouse model that is independent of RET-mediated signalling. In order to distinguish between acute and long-term effects of GDNF on the MPTP-induced damage to the nigrostriatal system, animals will be analyzed at two weeks and three months after MPTP intoxication.

Furthermore the experiments will be performed using $2x10^8$ transducing units of AAV-5 GFAP GDNF in order to primarily guarantee for ipsilateral restricted transgene expression, thus making it possible to use the contralateral side as clear internal control. Secondly, the very high amount of GDNF will prevent the miss of any signalling pathway, being initially activated in the presence of high amount GDNF. This strategy will answer the question if the presence of the RET receptor is mandatory for GDNF-mediated protection of the nigrostriatal system or if also other signalling pathways might play a role.

Questions:

5) Are GFP and GDNF after unilateral, striatal stereotaxic injection of $2x10^8$ AAV-5 GFAP GFP and AAV-5 GFAP GDNF equally expressed among the genotypes *DAT-Ret$^{lx/lx}$*, *DAT-Cre and Ret lx*?
6) Is it possible to detect any GDNF-mediated protective or regenerative effect in the nirgo-striatal system after MPTP intoxication, if the classical GDNF receptor RET is not present?

4. Methods

4.1 Molecular biology

Recombinant DNA techniques were performed according to the protocols described in Molecular Cloning Laboratory Manual, 2nd edition (Sambrook et al., 1989). Restriction endonucleases and DNA-modifying enzymes were used according to the manufacturer's instructions. Materials including chemicals, buffers, solutions, antibodies and primers are listed in chapter 4.9.

4.1.1 Propagation and preparation of plasmid DNA

Buffers and Media for bacterial culture

LB-medium: 10g/l tryptone, 5g/l yeast extract, 5g/l NaCl.
LB-plate: LB-medium, 2% (m/v) Agar.
SOC: 20g/l trypton, 5g/l yeast extract, 10mM NaCl, 10mM MgCl2, 10mM MgSO4, 20mM glucose

Plasmids were propagated in DH-5α maximum efficiency (Invitrogen GmbH, Karlsruhe) and SURE 2 super chemicompetent (Stratagene, La Jolla, USA) *E.coli* bacteria.

4.1.1.1 Bacteria culture conditions

All *E. coli* strains were cultured in LB-Amp liquid or solid (LB-Amp agar plates) medium, at 37ºC. For liquid culture, bacteria were incubated by shaking at 200-300rpm overnight.
Glycerolstocks for longterm storage were prepared by adding 800µl glycerol (80%) to 800µl overnight culture in a 2ml screw-cap vial. The vial was vortexed vigorously to ensure an even mixing of the bacterial culture and the glycerol, frozen in liquid nitrogen and stored at -80℃.

4.1.1.2 Heat shock transformation

Heat shock transformations of DH-5α maximum efficiency were performed according to the manufacturer's instructions. Briefly, after thawing 50µl bacteria suspension on ice, an appropriate amount of DNA (1-50ng purified plasmid DNA, 5-10µl of a ligation reaction) was added. Next, cells were gently mixed by finger tapping and incubated on ice for 30 minutes. After heat shocking in a water bath at 42℃ for 45s ec, cells were placed on ice for 2min. Next cells were supplemented with 450µl of SOC medium and incubated at 37℃ for 60min while shaking at 300rpm. 250µl of the suspension were then spread on LB-amp selective plates and incubated at 37℃ overnight. SURE 2 Supercompet ent were transformed in a similar manner: 40µl of bacteria suspension were pretreated by adding 0.8µl β-mercaptoethanol followed by 10min incubation on ice. An appropriate amount of DNA (1-50ng purified plasmid

DNA, 5-10µl of a ligation reaction) was added, the heat shock was carried out at 42℃ for 30sec. Cells were supplemented with 360µl SOC medium then 250µl of this suspension spread on LB-amp plates. The resulting single colonies were analyzed via colony PCR or used to inoculate LB-Amp liquid cultures.

4.1.1.3 Plasmid mini preparation

Buffers for plasmid mini preparation

Resuspension buffer: 50mM Tris-HCl; 10mM EDTA; 100µg/ml RNaseA; pH 8.0
Lysis buffer: 200mM NaOH; 1% SDS
Neutralization buffer: 3.0M potassium acetate, pH 5.5

An alkaline lysis method (modified according to Sambrook and Russel 2001) was used to extract the plasmid DNA from transformed cells by using the QIAprep Spin Miniprep Kit according to manufacturer's instructions. Five ml of LB-amp medium were inoculated with a single colony picked from a freshly streaked selective LB-Amp agar plate and incubated by shaking at 200-300rpm at 37℃. One ml overnight culture was centrifuged (13.000rpm, 5min, 4℃) in a micro centrifuge and the supernatant was discarded. Pelleted bacterial cells were dissolved completely in 300µl resuspension buffer (P1). 300µl lysis buffer (P2) were added, followed by inverting the tube 4-6 times and incubating for 5min at RT. After adding chilled 300µl neutralization buffer (P3), the lysate was mixed by inverting the tube 4-6 times and centrifuged (13.000rpm, 10min, 4℃). The supernatant was transfered to a fresh tube containing 600 µl room-temperature isopropanol, mixed and centrifuged immediately (13.000rpm, 30min, 4℃). The supernatant was decanted carefully and the DNA pellet washed with 500µl of 70% EtOH and centrifuged again (13.000rpm, 10min, 4℃). The supernatant was decanted and the pellet air dried for 5-10min then resuspended in 20µl TE buffer or sterile ddH20.

4.1.1.4 Plasmid Midi, Maxi and Mega preparations

To obtain large amounts of plasmid DNA, bacteria cultures were grown in 2x LB-Amp medium and midi, maxi and mega preparations were performed using QIAfilter® Plasmid Kits according to manufacturer's instructions**.** A preculture of 1.5ml was used to inoculate 1.5l of 2x LB-Amp medium to produce ~ 1.0mg plasmid DNA required for the preparation of viral vectors.

4.1.2 Isolation of genomic DNA from mouse tail biopsies

TENS buffer for isolation of genomic DNA

TENS buffer: 100mM Tris-HCl, 5mM EDTA, 200mM NaCl, 0.2% SDS, pH 8.5

Mouse tail biopsies of 0.5cm were placed in 1.5ml reaction tubes and incubated overnight in a thermomixer at 650rpm and 56℃ in 0.5ml TENS buff er containing 0.5mg/ml Proteinase K. Samples were then shifted to 90℃ to inactivate the Proteinase K. Samples were centrifuged (13.000rpm, 5min, 10℃), then 400µl of the supernat ant transfered to a fresh tube containing 500µl room-temperature isopropanol, then mixed and centrifuged immediately (13.000rpm, 5 min, 10℃). The supernatant was decanted carefully. The DNA pellet was washed with 100µl of 70% EtOH and centrifuged again (13.000rpm, 5min, 10℃). The supernatant was discarded completely; the pellet was dried for 15-20min at 42℃ then resuspended in 50µl TE buffer.

4.1.3 DNA precipitation

If increased purity and/or the concentration of DNA in the final solution were required, a precipitation step was included after the DNA extraction procedures. The samples were mixed with 100% isopropanol (70% of the original volumes) and 3M Na-acetate (10% of the original volumes). The samples were centrifuged for 20min at 4ºC, then the supernatant discarded. The DNA pellets were rinsed with 70% EtOH and centrifuged again. EtOH was carefully removed and the precipitates were air-dried at RT for 5-10min. The DNA pellet was resuspended in desired volume of ddH_2O or TE buffer.

4.1.4 PCR

PCR (polymerase chain reaction) was used to test plasmids for cDNA insertion and to add restriction sites, necessary for cloning of DNA fragments into respective plasmids, as well as epitope sequences to the cDNA by using corresponding primers. Prior to the amplification of the DNA sequences of interest, optimal PCR conditions were selected by varying the concentration of MgCl2, the amount of template DNA, annealing temperatures (50-60℃) and the use of additives (DMSO or glycerol). A standard PCR reaction mix typically contained:

reagent (stock concentration)	volume (final concentration)
template (cDNA/plasmid or genomic DNA)	variable (20-200ng)
10x PCR buffer	2.5µl
dNTP mix (10mM)	0.5µl
forward primer (10µM)	1.0µl
reverse primer (10µM)	1.0µl
Taq-polymerase (5U/µl)	0.25µl
ddH2O	ad 25µl

The amplification was performed in Mastercycler Gradient (Eppendorf) or DNA engine PTC-200 (MJ Research) thermocyclers using the following cycling conditions:

step	temperature	duration	number of cycles
initial denaturation	95℃	5min	1
denaturation	95℃	45sec	
primer annealing	55℃	1min	30
elongation	72℃	1min	
final elongation	72℃	10min	1

The amplification products were analyzed on 1% agarose gel by DNA electrophoresis. Amplified fragments designed to be inserted into plasmids were purified from the gel using a gel extraction kit.

4.1.5 DNA restriction, electrophoresis, gel extraction

Buffers and solutions for agarose gelelectrophoresis

TAE buffer : 40mM Tris/Acetat; 1mM EDTA; pH 8.0
6x TAE loading dye: 30% (v/v) glycerol, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) xylenol cyanol
Agarose gel: 0.5% - 2% Agarose in TAE, 0.001% Ethidiumbromide

Preparative restriction digests of 5-10µg of plasmid DNA were used to either obtain linearized plasmid vectors or to release cDNA fragments from plasmids. Analytic digests to test newly generated plasmid constructs were performed with 100-200ng plasmid DNA. Appropriate endonucleases in corresponding buffers (Fermentas) were mixed with DNA and left overnight at the temperature specified for each enzyme in the instruction manual of Fermentas. Analysis of the DNA size and purification of DNA fragments were performed by

agarose gel electrophoresis. DNA samples were mixed with 6x DNA loading buffer and ddH_2O to reach a final volume of 15-20µl and separated on an agarose gel in 1x TAE buffer at 100V. A DNA ladder (Fermentas) of appropriate range was used as size standard. The DNA bands were visualized in UV-light using a BIO-VISION™ fluorescence documentation system. DNA extraction after gel electrophoresis was performed following the QIAquick Gel Extraction Kit protocol. The concentration of DNA in the final solution was measured with a Nanodrop spectrophotometer at 260nm.

4.1.6 Cycle sequencing of PCR-amplified DNA

Cycle sequencing was performed using the services of StarSEQ GENterprise GmbH. 50-700ng of template (cDNA/plasmid DNA) and 1µl of an appropriate 10µM primer solution were sent to StarSEQ for sequencing with an ABI 3730 capillary sequencer based on the chain termination method developed by Frederick Sanger. Sequence analysis and alignment with reference cDNA were performed using Sequencer software.

4.1.7 Quantitative real-time PCR (qPCR)

qPCR was performed using the intercalating fluorescent dye SYBR® Green. In a first step total RNA was isolated out of mouse tissue using the “RNeasy Mini Kit” (Qiagen) according to manufacturer’s instructions. RNA concentration was determined with a Nanodrop spectrophotometer (Nanotech). RNA was digested by RQ1 RNase Free DNase (Promega) and protected against RNases by adding 20U of RNase Inhibitor RNasin (Promega). 2.5ug of total RNA was used for reverse transcriptase PCR (M-LV; Promega). cDNA was diluted 1:5. HPLC grade water was used for all reactions including template dilutions. qPCR reactions were prepared using “ABsolute™ QPCR SYBR® Green ROX Mix” (ABgene). Primers are listed in the material section.

reagent (stock concentration)	volume (final concentration)
template (cDNA derived from RNA)	2.0µl
SYBR® Green ROX Mix	12.5µl
forward primer (5µM)	0.35µl (70nM)
reverse primer (5µM)	0.35µl (70nM)
HPLC-grade H_2O	9.8µl

qPCR was performed in a Stratagene Mx3000P Realtime device (Stratagene) using the following cycling conditions:

step	temperature	duration	number of cycles
enzyme activation	95℃	15min	1
denaturation	95℃	15sec	40
primer annealing	primer T_m	30sec	
extension	72℃	30sec	
melting curve	95℃	30sec	1
	60℃	30sec	
	60-95℃	in 0.5℃ steps	

Fluorescence is detected and measured in real time, and its geometric increase corresponding to exponential increase of the product is used to determine the threshold cycle (C_T) in each reaction. The C_T value is characterized as the cycle in which the increasing fluorescence is significantly higher than the background fluorescence for the first time. Mouse Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as reference gene. Quantitative real time PCR analysis was carried out using the $2^{-\Delta\Delta Ct}$ method.

4.1.8 Plasmid construction: Cloning into AAV-plasmid

Construction of AAV vectors has been described previously (Kügler, et al., 2003). The pAAV-human-ß-actin-EWB, pAAV-no promoter-TB-EWB, pAAV-GFAP-EWB, pAAV-hsyn1-EWB and pAAV-hsyn1-GDNF-WB plasmids were kindly provided by Dr. Sebastian Kügler. In order to produce the pAAV-GFAP-GDNF-WB vector the pAAV-hsyn1-GDNF-WB plasmid was used as backbone. The hsyn1 promoter cDNA was substituted for the GFAP promoter cDNA coming from the pAAV-GFAP-EWB donor plasmid. The donor plasmid pAAV-GFAP-EWB was subjected to *Mlu*I/*Age*I restriction digest to release the GFAP promoter cDNA. The hsyn1 cDNA fragment was also removed from pAAV-hsyn1-GDNF-WB via *Mlu*I/*Age*I restriction digest. After a DNA precipitation step the *Mlu*I/*Age*I restriction sites were ligated at a 1:3 molar ratio (16℃ over night).

reagent (stock concentration)	volume (final concentration)
vector (4.7kb)	1.0µl (= 100ng)
insert (2.3kb)	12.0µl (= 300ng)
10x ligase buffer (Fermentas)	2.0µl
Ligase (Fermentas) 1U/µl	1µl
ddH_2O	4µl

In order to produce the pAAV-no promoter-TB3-EWB control vector expressing nothing, two transcription blocking elements (TB) were inserted into the pAAV-no promoter-TB-EWB backbone plasmid. The donor plasmid pAAV-no promoter-TB-EWB was subjected to *Xba*I restriction digest to release the TB cDNA and independently to a *Nhe*I restriction digest to linearize the backbone plasmid. After dephosphorylation of the linearized backbone plasmid (calf intestine alkaline phosphatase 1U (Fermentas), 30min at 37℃) the compatible *Xba*I/*Nhe*I restriction sites were ligated at a 1:3 molar ratio (16℃ over night) to inhibit religation and stop the reaction by DNA precipitation.

reagent (stock concentration)	volume (final concentration)
vector (5.0kb)	2.5µl (1:10 dilution= 100ng)
insert (0.2kb)	12.0µl (= 300 ng)
10x ligase buffer (Fermentas)	2.0µl
Ligase (Fermentas) 1U/µl	0.5µl
ddH_2O	3µl

SURE 2 Supercompetent *E. coli* cells were heat-shock transformed with the reaction mix (5-10µl of ligation mix) and spread on LB-Amp agar plates. Colonies were picked and screened for the presence of the insert via mini-preparation and different restriction digests.

4.2 Cell culture

The primary cortical rat culture was used to measure GDNF in the supernatant by ELISA after infection with AAV-5 GFAP GFP and AAV-5 GFAP GDNF and the human embryonic kidney 293 FT (HEK293FT) cells to check for protein expression after transfection of different plasmids by Western Blot.

4.2.1 Preparation of rat cortical neurons

To obtain primary cortical culture, cortexes were dissected from Wistar rats at E 18 (embryonic day 18) and further processed for establishing dissociated cell cultures as previously described (de Hoop et al. 1993). This primary culture consisted of a mixture of neurons and astrocytes and was kindly provided by Dr. Sebastian Kügler.

4.2.2 Culture conditions, transient transfection

HEK293FT cells were cultured in 75cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37℃ in a humidified atmosphere of 95% air and 5% CO_2 in a cell culture incubator. Cells were passaged at 90% confluence one to two times per week: Under sterile conditions, cell culture

medium was removed and cells were washed with PBS, before 1ml of trypsin/EDTA solution was added. Cells were incubated at 37℃ until they could be easily detached by tapping the flasks. The cells were thoroughly resuspended in 9ml cell culture medium to obtain a single cell suspension. 1ml of the suspension was transferred to a new flask containing 9ml of fresh medium. With this 1:10 dilution cells reached confluence after 4-6 days. The cell number was determined via light microscopy in a counting chamber. To perform cell culture experiments cells were plated at an appropriate density in 6-well or 12-well cell culture plates. Transient transfection of HEK293FT cells with 1µg of the target vector was carried out in 6-well plates using Metafectene transfection reagent (4µl) according to manufacturer's instructions.

4.2.3 Viral infection

For AAV infections 250,000 rat cortical neurons were plated in a 24-well plate. One week later the volume of medium was reduced from 750µl to 250µl and AAV in concentrations $1x10^8$ to $8\ x10^8$ infectious virus particles was added to the cells. 24 hours later the volume of medium per well was increased again to 750µl. 3 days post infection the cells received fresh medium and 1 week post infection cells expressed the transgene (controlled by GFP control expression) in a sufficient amount to collect the cells and supernatant for further experiments (ELISA, Immuno Blot).

4.3 Protein biochemistry

This section describes all protein biochemistry related work performed in this study.

4.3.1. Preparation of cell culture lysates

RIPA Buffer for protein extraction

RIPA buffer: 50mM Tris, pH 8.0, 0.15M NaCl, 0.1% SDS, 1.0% NP-40, 0.5% Na-Deoxycholate, 2mM EDTA, Complete™ Protease Inhibitors, pH 7.4

Medium was aspired from culture dishes and cells were washed with PBS. Cells were scraped in PBS and, in order to decrease the volume, cells were centrifuged for 8min at 6000 rpm on 4℃. The resulting pellet was resuspended in an appropriate volume of Ripa lysis buffer containing Complete protease inhibitor cocktail and left on ice for 30min for lysis to occur. After centrifugation for 10min at 13000rpm at 4℃, the supernatant was taken and the pellet containing insoluble cell debris was discarded. For preservation of proteins over a longer period of time aliquots were frozen and kept at -20℃.

4.3.2 Preparation of tissue lysates

After PBS-perfusion, murine tissue was rapidly dissected and kept in 1.5ml reaction tubes on dry ice until Ripa buffer was added or until addition of RLT buffer (RNeasy Mini Kit) in the case of RNA and protein extraction out of the same sample. Cells were lysed using a Precellys 24 homogenisator. The lysates were cleared twice from cell debris by centrifugation (10min, 13.000rpm at 4℃).

If RNA and protein were wanted to be extracted out of the same sample, total protein was isolated via aceton precipitation from the RLT buffer lysates using the RNeasy Mini Kit and following the manufacturer´s instructions. Total protein was resuspended in 0.23M Tris-HCl pH 6.8, 50% glycerol and 5% SDS.

4.3.3 Determination of protein concentration

Protein concentration was determined using the coomassie blue G-based Bio-Rad Protein Assay. A 1:5 dilution of the assay reagent stock solution was prepared in ddH_2O. A standard curve was prepared using bovine serum albumine (BSA) with concentrations ranging from 0-12µg/µl in ddH_2O. Triplets of known protein concentrations were added into 96-well plates (50µl) together with samples of unknown concentrations (49µl of ddH_2O and 1µl of protein lysate). The assay reagent was added to the final volume of 200µl. After incubating for 5min at RT, protein concentrations were assayed by measuring absorbance at 595nm using a Mithras LB 940 plate reader, equipped with Mikro Win software 2000. If RNA and protein were wanted to be extracted out of the same sample protein concentration was measured and calculated using the DC Protein Assay (BIO-RAD), tolerating a high SDS concentration and according to manufacturer's instructions.

4.3.4 SDS-PAGE

Buffers for protein separation on SDS polyacrylamide gels

5x Laemmli buffer: 10% Glycerol, 20% SDS (10% (w/v)), 12.5% 0.5M Tris-Cl, pH 6.8, 5% β-Mercaptoethanol, 5% Bromphenol blue (1% (w/v))
10x Running Buffer: 250mM Tris/HCl; 1.92M Glycine; 2% SDS, pH 8.3

For SDS-PAGE the "Protean III mini-gel system" (Bio-Rad) was used. Gel components were mixed at the required percentage of acryl amide according to the tables for running and stacking gel. Samples in 5 x Lämmli buffer containing 20-50µg of protein were boiled for 5min on 95℃ and cooled down on ice immediately aft er incubation on 95℃, thus preventing renaturation of proteins. After brief centrifugation samples were subjected to gel

electrophoresis at 120V constant voltage in running buffer. A prestained marker was used as a protein size standard.

Running gel (all volumes in ml)

	10%	12%
30% Acrylamide	1.7	2
4x running gel buffer	1.3	1.3
10% APS	0.025	0.025
TEMED	0.025	0.025
H_2O	2.1	1.75

Stacking gel (all volumes in ml)

	7.5%
30% Acrylamide	0.625
4x stacking gel buffer	0.625
10% APS	0.013
TEMED	0.013
H_2O	1.25

4.3.5 Immuno blot

Buffers and Solution for protein transfer

Transfer buffer: 25mM Tris, 192mM Glycine, 20% methanol, pH 8.3
Ponceau solution: 0.1% Ponceau-S in 5% Acetic acid
TBS: 10mM Tris/HCl, 150mM NaCl, pH 7.5
TBS-T: TBS with 0.1% (v/v) Tween20
Blocking solution: 5% (w/v) skim milk in TBS
Stripping buffer: 0.2M Glycine, 0.5M NaCl, pH 2.8

Separated proteins on SDS-PAGE were transferred to a Hybond nitrocellulose membrane (Amersham). Nitrocellulose membrane, Whatmann paper and foam pads were first soaked in 1x Transfer buffer. A sandwich was built up on the cathode containing 1xfoam pad, 1x3 mm paper, gel, PVDF membrane, 1x3mm paper, 1 x foam pad. The anode was fitted and the transfer performed for 1h at constant voltage (100V). The transfer was checked by dying a membrane with Ponceau-S solution (0.1% Ponceau-s in 5% Acetic acid).

The transfer membrane was placed for 1h in blocking solution (TBS; 5% (w/v) skim milk) at room temperature. Subsequently the membrane was incubated with the primary antibody, appropriately diluted in 1% skim milk in TBS at 4℃ over night. The next day, the membrane was washed 3x10min with TBS-T and incubated with an adequate HRP-coupled secondary antibody, appropriately diluted in TBS, for 1h at room temperature. Membrane was washed again 3x10min prior to incubation with the Chemiglow chemiluminescent substrate (Alpha Innotech) for 1min. Bound proteins were visualized using the AlphaImager video imaging device equipped with Fluor Chem 8900 software (Alpha Innotech), which was also used for signal quantification.

4.4 Adeno-associated virus production

AAV-5s were produced by Dr. Sebastian Kügler according to standard protocols of the CMPB viral vector service platform (Diss Manuel Joaquim Marques Garrido 2008). Briefly, vectors were propagated in AAV-293 cells (Stratagene) using pDG2 as helper plasmid. Viruses were concentrated and purified by an iodixanol step gradient procedure. Each virus was further purified and concentrated by collecting the “peak” elution phase on Äkta-FPLC (Amersham) system using HiTrap^{TM} Heparin HP 1 columns (GE Healthcare). Further concentration and purification of rAAV-5 particles was performed by dialysis using Slide-A-Lyzer (molecular weight cut-off = 10,000). The final virus solution was subjected to real-time PCR (RT-PCR) quantification of the rAAV-vector genome particle titre. Purity of the vectors was determined by SDS-gel electrophoresis and infectious titre by transduction of cultured primary cortical neurons. The genome particles to transducing units (TU) ratio typically was 25:1 - 35:1 and was calculated as 30:1. The AAV vectors were aliquoted and stored at -80℃ until used. During all procedures with AAV vectors, 0.5% SDS solution in water was used for disinfection. The transducing titres of AAVs used in this study were $3x10^8$/µl for AAV-5 no-promoter-TB_3 GFP, $5x10^8$/µl for AAV-5 GFAP GFP, $0.9x10^8$/µl for AAV-5 SYN GFP, $1.9x10^8$/µl for AAV-5 GFAP GDNF, $1.2x10^8$/µl for AAV-5 SYN GDNF.

4.5 Animal work

This section describes all animal work performed in this study.

4.5.1 Animal housing and strains

Animal experiments were carried out on mice, in accordance with the German Animal Welfare Act (TierSchG) and approved by Regierungspräsidium Hannover. Male and female mice were obtained from Charles River or were breed in the animal facilities of the Universitiy of Goettingen and the Centre for Molecular Neurobiology Hamburg (ZMNH). Mice were maintained in a temperature/humidity-controlled environment under a 12hr light/dark cycle

with free access to food and water. The following mouse strains were used: C57Bl/6-J, *Ret* ^{lx}, *DAT-Cre* and *DAT/Ret* $^{lx/lx}$. To selectively disrupt the gene encoding for Ret in DA neurons Kramers group used mice with floxed allele of Ret *(Ret* ^{lx}) in combination with *DAT-Cre* mice, resulting in Ret deficiency in DA neurons (*DAT/Ret* $^{lx/lx}$) (Kramer et al., 2007).

4.5.1.1 Genotyping

Genotyping of transgenic and knock-out mice was performed by PCR using genomic DNA extracted from tail biopsies as template with appropriate primer pairs by Dr. Edgar Kramer at the ZMNH.

4.5.2 Surgery: stereotaxical injections

Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine solution (100 or 5 mg/kg bodyweight, respectively) and placed with flat skull position in a stereotaxic frame (WPI). Murine eyes were protected from dehydration by Bepanthen creme (Bayer). A mineral oil-sealed glass capillary was fitted into a Nanoliter2000 microinjector and filled with the required volume of virus preparation. After slitting the scalp with a scalpel, a hole of 1 mm diameter was drilled into the skull to insert the tip of the capillary to the respective coordinates relative to bregma. Injections were made as two deposits along the needle tract at the coordinates listed below.

Structure (total inject. vol)	anterior	lateral (right)	ventral (from skull surface)
Striatum 2µl = $2x10^8$	+0.0mm +0.9mm	-2.2mm -1.5mm	3.0; 4.0mm

After two minutes injection was started with a rate of 250nl/min. The capillary was left in place for additional 2min before moving to the deeper coordinates in the same needle tract and 5min before withdrawal.

As post-operative treatment, mice were kept warm with a heating pad and received subcutaneously a 1ml deposit of a 1:1 mixture of glucose (5% Braun) and NaCl (0.9% Diaco) and were provided with wet mashed food. The wound was closed using Histoacryl (Braun). As pain therapy the mice received Novaminsulfon (Ratiopharm) in the drinking water in a concentration of 3.2mg/ml.

4.5.3 Subchronic MPTP treatment

Two weeks after unilateral AAV delivery to the *striatum*, mice were treated either with NaCl or MPTP hydrochloride. MPTP was administered at a dose of 30mg/kg free base solved in NaCl intraperitoneally at 24h intervals for five doses.

4.5.4 Tissue preparation and processing

10x Stock Solution of PBS buffer

10x PBS: 1.37M NaCl, 82mM Na_2HPO_4, 15mM KH_2PO_4, 2 mM KCl, pH 7.4

Mice were sacrificed using CO_2. In order to remove blood from the brain mice were transcardially perfused with PBS and in a second step with 4% paraformaldehyde (PFA)/ PBS using gravitational force, thus starting the tissue fixation process. The brains were removed rapidly, immersion fixed in 4% PFA/PBS overnight and cryoprotected in 30% sucrose until tissue was heavier than the fluid at 4℃. In a next step the brains were rapidly frozen in isopentane on dry ice. Complete sets of serial coronal sections were cut through *striatum* and SNpc at 30µm on a cryomicrotome and collected in 24 well plates in PBS with 0.1% Na-Azide.

4.5.5 Behavior

Animal behavior regarding locomotor function, as a further important analysis point, was followed by open field, rotarod and rotation performance and the tight rope test.

4.5.5.1 Open Field

The locomotor activity of the AAV-injected and control cohorts was examined by monitoring their horizontal and vertical movements in an open field to determine whether there was a difference between untreated control animals and the two MPTP and AAV-injected groups (GFP/GDNF). The motor activity of mice was increased by amphetamine to elucidate more clear differences. Animals were surveyed in a 50 x 50cm arena by using the “Videomot2” video tracking system (TSE Systems) during the whole experiment. Rearing was detected by infrared beams. The tracking arena was divided into a “center” and a “border” zone using the Vidoemot2 software. Eleven to fourteen days after MPTP injection, mice were placed separately once into the arena after having received the amphetamine injection intraperitoneally (5mg/kg body weight). During a tracking period of 60min, the time, distance, number of visits, latency, number of rearing and rear time in each area was recorded. At the end of the experiments, mice were returned to their housing cages.

4.5.5.2 Rotarod

A rotarod test was used to assess the effect of MPTP and AAV injection on motor performance and coordination of the mice using a gradually accelerating rotarod apparatus (47600 ROTA-Rod for Mice, UGO BASILE) set to accelerate from 10 to 40rpm over 180s. Two training sessions were performed at a constant speed of 10rpm on two different days before starting the measurements. Before and after MPTP administration the animals performed 3 times on the gradually accelerating rotarod within one day. The data from the rotarod experiments are expressed as the sum of seconds the animals were able to remain on the rotarod before falling (latency to fall). The performance was aborted at the latest time point of 400s (starting from 180s stable at 40rpm).

4.5.5.3 Tight rope

A tight rope test was used to further analyze the motor performance of the mice before and after MPTP and AAV injection in comparison to untreated animals. During a training period the mice had to climb from a central position between two platforms, positioned 30cm above the ground on a 60cm long tight rope to one of these platforms in a reasonable time period.
For animals that performed well enough in these training sessions, the time they needed to reach one of the platforms in six rounds of performance was recorded one day before and after NaCl / MPTP treatment.

4.5.5.4 Rotation

In order to reveal side preferences regarding motor performance of animals treated systemically with MPTP and unilateral with AAV compared to control groups, animals performed the rotation test. In each trial 4 mice were placed for ten minutes in 4 buckets (height: 30cm; radius: 13cm) in order to let them adapt to the new environment. After having injected amphetamine (5mg/kg body weight) intraperitoneally to induce the rotating behavior, left and right turns were counted manually every 5min for 1min over a time period of 30min. In order to ascertain individual side preferences the mice were measured once before MPTP treatment and one and two weeks afterwards. The data from the rotating experiments are expressed as quotient of left to right turns after calibration to the pre MPTP data.

4.5.6 Histology

Immunohistochemistry of 30µm cryosections was performed free-floating.

4.5.6.1 Nissl staining

Nissl staining solution:
buffer solution: sodium acetate 7g, glacial acetic acid (100%) 2ml in 1000ml aqua bidest.
stock stain solution: thionin acetate 1g in 100ml aqua bidest.
working stain solution: stock solution 45ml and 455ml buffer solution

The Nissl method uses basic aniline to stain RNA blue, and is used to highlight important structural features of neurons. The Nissl substance appears dark blue due to the staining of ribosomal RNA, giving the cytoplasm a mottled appearance. Following the 3,3'-Diaminobenzidine (DAB) immunohistochemical staining, sections were rinsed in ddH_2O, stained in a solution containing 0.1% thionin, pH 5.5 for 7 minutes, rinsed in ddH_2O, pre-dehydrated in 70% and 90% ethanol (2 minutes each) and differentiated in 96% ethanol for 5 minutes, dehydrated in 100% isopropanol (5min), cleared in xylene (3x 5min) and mounted with xylene-based medium (DPX Mountant for histology, Fluka).

4.5.6.2 Immunohistochemistry

Buffers for histology stainings

Acetate imidazole buffer: 1ml sodium acetate 1M, 1ml imidazole 0.2M to 18ml aqua bidest.
PBS: 137mM NaCl, 8.2mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCl, pH 7.4
TBS: 0.1M Tris, 150mM NaCl, pH 7.4
Dilution Media (DM): 4.1g NaCl, 5.2g Trizma, 350µl Triton X-100 in 700ml aqua bidest.
Mowiol-mounting medium: 24% w/v Glycerol, 0.1M Tris-base pH 8.5, 9.6% w/v Mowiol 4.88 and 2.5% w/v DABCO
Sodium periodate: 2.13g sodium periodate in 100ml TBS

Antibodies and their dilutions are listed in the appendix section. All immunohistochemical stains were performed according to the following general protocol: After washing 3x 5 min with washing buffer, sections were pretreated with a 10% Methanol, 1-3% H_2O_2 TBS solution for endogenous peroxidase quenching, if necessary, washed again and incubated in blocking solution for 1h at room temperature. Incubation with the primary antibody 24-48h was followed by 1-2h incubation with the secondary antibody, washing and mounting.
For fluorescent (multiple) labeling fluorochrome-conjugated secondary antibodies were used or in order to further increase the signal biotinylated secondary antibodies combined with an

avidin–biotin complex (ABC) Vector kit and the TSA™ Plus System (PerkinElmer) based on tyramide amplification reagent. TSA can be easily integrated after the addition of horseradish peroxidase (HRP), which is used to catalyze the deposition and covalent binding of fluorescent labeled tyramide. For light microscopy the antibody reaction was visualized by DAB using ABC Vector kit. Detailed protocols are listed below (Table 4.1).

Table 4.1: Protocols for light and fluorescent microscopy.

Light microscopy				**Fluorescent microscopy**	
Staining type	DAB for SNpc	DAB for striatum	GDNF staining	without signal amplification	signal amplification
Wash	3x 5 min in TBS	3x 10 min in TBS	3x 5 min in DM		3x 10 min in TBS
Pre-treatment	Quenching endogen peroxidase (5min 3% H_2O_2/10% methanol)	Quenching endogen peroxidase (15min 1% H_2O_2/40% methanol)	Quenching endogen peroxidase (20min sodium periodat)		Quenching endogen peroxidase (15min 1% H_2O_2/40% methanol)
Wash	see above	see above	3x 10 min in DM	3x 5 min in 0,3% Triton-X100 in PBS	see above
Block	5% NGS in TBS	10% NGS, 3% BSA, 0,1% Triton X-100 in PBS	3.3 % NHS, 2% BSA in DM	10% NGS, 0,3% Triton X-100 in PBS	10% NGS, 3% BSA, 0,1% Triton X-100 in PBS
1st Ab	diluted in TBS / 2% NGS 48hs/4℃	diluted in 10% NGS, 3% BSA, 0,1% Triton X-100 in PBS 48hs/4℃	1% NHS, 1% BSA, 0,4% Triton X-100 in PBS 48hs/4℃	diluted in 1% NGS, 0,3% Triton X-100 in PBS 24hs/4℃	diluted in 10% NGS, 3% BSA, 0,1% Triton X-100 in PBS 48hs/4℃
Wash	see above	see above	6x 10 min in DM	3x 5 min in 0,3% Triton-X100 in PBS	see above

2nd Ab	biotin-conjugated, diluted in TBS 2% NGS 1h/room temperature	biotin-conjugated, diluted in 10% NGS, 3% BSA, 0,1% Triton X-100 in PBS 2hs/room temperature	biotin-conjugated, diluted in 1% NHS, 1% BSA in DM 1h/room temperature	fluorochrome-conjugated, diluted in 1% NGS, 0,3% Triton X-100 in PBS 1h/room temperature	biotin-conjugated, diluted in 10% NGS, 3% BSA, 0,1% Triton X-100 in PBS 2hs/room temperature
Wash	see above	see above	see above	3x5 min in 0,3% Triton-X100 in PBS	see above
Detection	ABC-Kit standard (1h)/DAB (15min)	ABC-Kit elite (2hs)/DAB (10min)	ABC-Kit standard (30 min)/ DAB (10min)	fluorescence	ABC-Kit elite (2hs)/ TSA^{TM} Plus System (10min)
Wash	see above	see above	3x 10 min acetate imidazole		see above
Mounting	DPX mount after Nissl staining	DPX mount after dehydration	DPX mount	Mowiol/DABCO	Mowiol/DABCO

4.6 Microscopy

Fluorescent images were acquired using by an inverted fluorescence microscope (Leica DMI6000B) equipped with 2.5x, 10x, 20x, 40x, 63x dry and 63x water objectives, Leica FX350 (black/white) and FX450 (color) digital cameras digital cameras and the Leica Advanced Flurorescence Software. Transmitted light images were acquired by an Axioskop 2 (Zeiss) equipped with 2.5x, 10x, 20x, 40x dry and a 63xoil-immersion objective, a MicroFire camera (Optronics) and the StereoInvestigator software.

4.7 Quantifications

All quantifications were performed blinded for treatment.

4.7.1 Stereology

Stereological counts were carried out with the optical fractionator method (StereoInvestigator, MBF Bioscience, Magdeburg). The criterion for counting an individual cell was its presence either within the counting frame, or touching the right or top frame lines

(green), but not touching the left or bottom lines (red). The total number of cells was then determined by the StereoInvestigator program. The number of SNpc dopaminergic neurons was determined by counting Nissl-positive and TH-immunoreactive neurons using an oil-immersion 63x objective (Axioskop 2, Zeiss) a counting frame of 50x50 µm and a grid size of 100x100 µm, while the region of interest was confined to the SNpc.

4.7.2 Optical density

To quantify TH-IR terminals in the *striatum*, five coronal sections between bregma +1.10 and −0.10mm throughout the *striatum* were stained for TH with DAB and ABC Elite kit as described above. For every section, three pictures were acquired. To automatically delineate the fibres and to increase the signal-to-noise ratio, the images were first thresholded, binarized and the optical density of photographs evaluated with ImageJ software. Striatal values were normalized by subtracting the background staining, measured in the cortex of the same section. For the GDNF-Ret signaling study the TH- and DAT-positive fibre density in the *striatum* will be analyzed by Dr. Edgar Kramer as previously described (Kowsky et al. 2007).

4.7.3 High performance liquid chromatography (HPLC)

For the measurement of catecholamines by HPLC the mice were sacrificed 14 and 90 days after the last MPTP injection by fast cervical dislocation, because of the instability of catecholamines after death. The striata were rapidly dissected, frozen, and stored at −80℃. Tissue samples were mechanically crushed (Precellys 24) in 50µl of 0.1M perchloric acid per milligram of striatal tissue. After centrifugation (15,000×*g* for 10min at 4℃), 20µl of supernatant was injected onto a C18 reverse-phase HR-80 catecholamine column (ESA, Bedford, MA). DOPAC and HVA were quantified by HPLC with electrochemical detection (Dionex Ultimate 3000 machine, Dionex). The mobile phase (pH 4,3) consisted of 57mM citric acid, 43mM sodium acetate, 0.1mM EDTA, 1mM octane sulfonic acid and 20% methanol. The samples were first separated on a chromatographic column (Dionex Acclaim C 18, 5µm, 2.1 x 150mm column, at 25˚C) and then electrochemically detected on a graphite electrode (Dionex ED50 elektrochemicaldetector with the following conditions: disposal carbon electrode at 0.8V, flow rate 0.2ml/min. DA, HVA and DOPAC (Sigma-Aldrich) standards of 1.5, 0.3 and 0.15µM were included in each HPLC run for creation of a standard curve. Data were collected and processed using the Chromeleon 6.60 software.

4.8 Statistics

Data are expressed as means ± SD. Statistical analysis was performed by ANOVA, followed by Tukey's post hoc test to compare group means with GraphPad Prism 4.0 (GraphPad Software). Significance levels were set at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

4.9 Materials

The listed instruments, consumables, chemicals, media, supplements and buffers for cell culture, enzymes, pharmaca, narcotics, kits, antibodies, bacterial strains, cell lines and mouse lines, primer and vector maps have been used in the submitted thesis. All supplier companies listed below are located in Germany, if not otherwise stated.

4.9.1 Instruments

Autoclave	
Systec 5075 ELV	Systec GmbH, Wettenberg
Bead grinder homogenizer	
Precellys 24	Peqlab, Biotechnologie, Erlangen
Cell-counting chamber	
Neubauer cell chamber	Glaswarenfabrik Hecht GmbH, Sondheim/Rhön
Centrifuges	
Sigma 1-15K Sigma 6K 15	Sigma-Aldrich Chemie GmbH, Taufkirchen
Centrifuge 5415D	Eppendorf Deutschland, Hamburg
Developing machine	
FMP 800 A	Fujifilm Europe, Düsseldorf
ECL-camera system	
AlphaImager EC	Alpha Innotec Corporation, San Leandro, USA
Electrophoresis chambers for agarose gels	
Mini Sub Cell GT	Bio-Rad Laboratories GmbH, München
Gel documentation	
BioVision Video documentation system	Peqlab Biotechnologie, Erlangen
Heat blocks	
Thermofixer comfort	Eppendorf Deutschland, Hamburg
HPLC	
UltiMate 3000	Dionex, Idstein
Incubators	

Sanyo CO_2-Inkubator MCO 18AIC	MS Laborgeräte, Wiesloch
Luminometer	
Mithras LB 940 plate reader	Berthold Technologies, Bad Wildbad
Microinjector	
Nanoliter2000 microinjector Micro4 smart controller unit	WPI (World Precision Instruments), Berlin
Microtomes	
Cryostat LEICA CM3050S	Leica Microsystems, Bensheim
Microscopes	
Axioskop 2	Zeiss, Göttingen
Leica DMI6000B	Leica Microsystems, Bensheim
pH meter	
CG843/14pH	Schott Instruments GmbH,
Pipets	
Biohit mLine (10, 20, 200, 1000 µl)	Biohit Deutschland GmbH, Rosbach v. d. Höhe
Gilson Pipetman (10, 20, 200, 1000 µl)	Gilson International B.V., Limburg-Offheim
Eppendorf Reference (10, 20, 200, 1000 µl)	Eppendorf Deutschland, Hamburg
Photometers	
NanoDrop 1000 Spectrophotometer	Thermo Fisher Scientific, Dreieich
Power supplies	
Power-Pac 300	Bio-Rad Laboratories GmbH, München
Power-Pac 3000	Bio-Rad Laboratories GmbH, München
Rotarod	
47600 Rota-Rod for mice	UGO Basile, Comerio VA - Italy
SDS-PAGE- and Blotting-Apparatus	
Mini-PROTEAN® II-Electrophoresis-System	Bio-Rad Laboratories GmbH, München
PROTEAN® II xi-Electrophoresis-System	Bio-Rad Laboratories GmbH, München
Shakers	
Incubation shaker Unitron	Infors HT, Einsbach
Roller Shaker "Assistent" RM5	Glaswarenfabrik Hecht GmbH, Sondheim/Rhön
Orbital platform shaker Rotamax 120	Heidolph Instruments GmbH & Co.KG, Schwabach
Vortex-Genie 2	neoLab Migge Laborbedarf GmbH, Heidelberg

Sterile bench	
HeraSafe HSP	Thermo Fisher Scientific, Dreieich
stereotaxic frame	WPI (World Precision Instruments), Berlin
Scales	
EW4200-2NM	Kern & Sohn GmbH, Balingen
ALC-210-4	Acculab, Bradford, USA
Thermocycler	
Mastercycler Gradient	Eppendorf Deutschland, Hamburg
MX3000P™ Real time PCR system	Stratagene, La Jolla, USA

4.9.2 Consumables

Gel blotting paper	
Type GB3000	Whatman GmbH, Dassel
Nitrocellulose membranes	
PROTRAN® Nitrocellulose transfer membrane	Whatman GmbH, Dassel
Cell culture plastic	
BD Falcon 6-/24-/48-/96-*well* cell culture plates	BD Biosciences, Heidelberg
Costar® cell culture bottles, 75 cm^2	Corning GmbH, Life Sciences, Wiesbaden
Nunclon Surface 96-*well* cell culture plate, flat bottom	Nunc, Langenselbold
Histology	
SuperfrostPlus® glass slides	Menzel-Gläser, Braunschweig
Surgery	
sterile scalpel blades, syringes, canules	B. Braun Melsungen AG, Melsungen

4.9.3 Chemicals and biochemicals

Acetic acid	Carl Roth GmbH, Karlsruhe
Acetone	Carl Roth GmbH, Karlsruhe
Acrylamide 2K-Solution (30%, Mix Acrylamide:Bisacrylamide; 37,5:1)	AppliChem, Darmstadt
Agarose	Peqlab, Biotechnologie, Erlangen
Ammonuim chloride	Sigma-Aldrich Chemie GmbH, Taufkirchen
Ammonium persulfate (APS)	Sigma-Aldrich Chemie GmbH, Taufkirchen
Ammonium sulfate	Merck, Darmstadt
Ampicillin	Sigma-Aldrich Chemie GmbH, Taufkirchen
BenchMark® Prestained Protein Ladder	Invitrogen, Karlsruhe
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, Taufkirchen
Bromphenol blue	Merck, Darmstadt

Chemiglow chemiluminescent substrate	Alpha Innotec, San Leandro, USA
Hydrochloric acid	Carl Roth GmbH, Karlsruhe
Citric acid monohydrate	Fluka, Neu-Ulm
Complete® Proteases-Inhibitor-Cocktail	Roche Diagnostics GmbH, Mannheim
DAB	Sigma-Aldrich Chemie GmbH, Taufkirchen
DABCO	Sigma-Aldrich Chemie GmbH, Taufkirchen
Desoxynucleosid-5'-triphosphate (dNTPs)	Invitrogen GmbH, Karlsruhe
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Taufkirchen
Dopac	Sigma-Aldrich Chemie GmbH, Taufkirchen
Dopamine	Sigma-Aldrich Chemie GmbH, Taufkirchen
DPX mounting medium	Fluka, Neu-Ulm
EDTA	Sigma-Aldrich Chemie GmbH, Taufkirchen
Ethanol, absolute extra pure	Merck, Darmstadt
Ethidium bromide	Sigma-Aldrich Chemie GmbH, Taufkirchen
GeneRuler® 1 kb-DNA-Ladder	Fermentas Life Sciences, St. Leon-Rot
GeneRuler® 100 bp-DNA-Ladder	Fermentas Life Sciences, St. Leon-Rot
Glucose	Sigma-Aldrich Chemie GmbH, Taufkirchen
Glucose oxidase	Sigma-Aldrich Chemie GmbH, Taufkirchen
Glycine	AppliChem, Darmstadt
Glycerol	Sigma-Aldrich Chemie GmbH, Taufkirchen
Guanidine isothiocyanate	Mobitec Molecular Biotechnology, Göttingen
Histoacryl	B. Braun, Melsungen
Homovanillic acid	Sigma-Aldrich Chemie GmbH, Taufkirchen
Hydrogen peroxide (30%)	Merck KGaA, Darmstadt
Immersion oil	Merck KGaA, Darmstadt
Imidazole	Fluka, Neu-Ulm
Isopropanol (molecular grade)	Sigma-Aldrich Chemie GmbH, Taufkirchen
LB Agar	Invitrogen GmbH, Karlsruhe
LB Broth	Invitrogen GmbH, Karlsruhe
Magnesium chloride	Fluka (Sigma-Aldrich)
Magnesium sulfate	Fluka (Sigma-Aldrich)
Metafectene transfection reagent	Biontex Laboratories, Martinsried
Methanol	Carl Roth GmbH, Karlsruhe
2-Methyl-butane	Carl Roth GmbH, Karlsruhe
Mineral oil	Fluka, Neu-Ulm

Mowiol 4.88	Calbiochem, Darmstadt
New born goat serum	Invitrogen GmbH, Karlsruhe
New born horse serum	Invitrogen GmbH, Karlsruhe
N,N,N',N'-Tetramethylethylendiamin (TEMED)	Carl Roth GmbH, Karlsruhe
NaCl solution (0.9%, steril, for injection)	B. Braun Melsungen AG, Melsungen
Naturaflor® Milk powder	Töpfer GmbH, Dietmannsried
Nickel ammonium sulfate	Fluka (Sigma-Aldrich)
NP-40	Sigma-Aldrich Chemie GmbH, Taufkirchen
O.C.T. compound	Sakura, Staufen
Paraformaldehyde (PFA)	Riedel-deHaën, Seelze
Ponceau S	Sigma-Aldrich Chemie GmbH, Taufkirchen
Potassium chloride	Merck KGaA, Darmstadt
Potassium dihydrogen phosphate	Merck KGaA, Darmstadt
Prestained SDS-PAGE Standard (Broad Range)	Bio-Rad, München
Saccharose	Carl Roth GmbH, Karlsruhe
Sodium acetate	Carl Roth GmbH, Karlsruhe
Sodium azide	Carl Roth GmbH, Karlsruhe
Sodium bicarbonate	Sigma-Aldrich Chemie GmbH, Taufkirchen
Sodium carbonate	Sigma-Aldrich Chemie GmbH, Taufkirchen
Sodium chloride	Carl Roth GmbH, Karlsruhe
Sodium deoxycholate	Sigma-Aldrich Chemie GmbH, Taufkirchen
Sodium dodecyl sulphate (SDS)	AppliChem GmbH, Darmstadt
Sodium hydrogen phosphate	Carl Roth GmbH, Karlsruhe
Sodium hydroxide	Carl Roth GmbH, Karlsruhe
Sodium 1-octanesulfonate monohydrate	Fluka (Sigma-Aldrich)
Sodium periodate	Fluka (Sigma-Aldrich)
Sodium vanadate	Sigma-Aldrich Chemie GmbH, Taufkirchen
ß-Mercaptoethanol	Fluka (Sigma-Aldrich)
Temed	AppliChem, Darmstadt
Thionine acetate	Sigma-Aldrich Chemie GmbH, Taufkirchen
Trichloracetic acid	Merck KGaA, Darmstadt
Triton X-100	Sigma-Aldrich Chemie GmbH, Taufkirchen
Tris	Carl Roth GmbH, Karlsruhe
Tris-HCl	Carl Roth GmbH, Karlsruhe
Trizma	Fluka (Sigma-Aldrich)

Tween® 20	Carl Roth GmbH, Karlsruhe
Xylenole cyanole	Carl Roth GmbH, Karlsruhe
Xylol	Carl Roth GmbH, Karlsruhe

4.9.4 Media, supplements and buffers for cell culture

Dulbecco's Modified Eagle Medium (DMEM)	PAN-Biotech, Aidenbach
Dulbecco's Phosphate Buffered Saline (DPBS),	Invitrogen GmbH, Karlsruhe
Fetale Calf Serum (FCS)	Invitrogen GmbH, Karlsruhe
Penicillin/Streptomycin	PAN Biotech, Aidenbach

4.9.5 Enzymes

Absolute™ QPCR SYBR® Green Rox Mix	Abgene, Epsom, UK
Complete™ protease inhibitors	Roche Diagnostics GmbH, Mannheim
DNase	Promega, Mannheim
Proteinase K (PCR grade)	Roche Diagnostics GmbH, Mannheim
Restriction endonucleases	Fermentas Life Sciences, St. Leon-Rot
M-MLV reverse transcriptase	Promega, Mannheim
RNasin ribonuclease inhibitor	Promega, Mannheim
T4 ligase	Fermentas Life Sciences, St. Leon-Rot
Taq Polymerase	Fermentas Life Sciences, St. Leon-Rot
Trypsin/EDTA	Invitrogen GmbH, Karlsruhe

4.9.6 Pharmaca and narcotics

Bepanthen eye and nose ointment	Bayer, Leverkusen
D-Amphetaminesulfate	Sigma-Aldrich Chemie GmbH, Taufkirchen
Ketamine solution Ketanest-S	Pfizer Pharma GmbH, Berlin
MPTP	Sigma-Aldrich Chemie GmbH, Taufkirchen
Novaminsulfonratiopharm	Ratiopharm, Ulm
Xylazine	Riemser, Greifswald

4.9.7 Kits

Vectastain® ABC Kit	Vector Laboratories Inc., Burlingham, USA
Biorad Protein Assay	Bio-Rad Laboratories GmbH, München
QIAfilter® Plasmid purification Kits	Qiagen GmbH, Hilden
QIAquick® Gel extraction Kit	Qiagen GmbH, Hilden
QIAquick® PCR purification Kit	Qiagen GmbH, Hilden
GDNF Emax® ImmunoAssay System	Promega, Mannheim
RNeasy Mini Kit	Qiagen GmbH, Hilden
TSA Plus Fluorescence System	PerkinElmer, Rodgau-Juegesheim

4.9.8 Antibodies

Primary antibodies	
GDNF, polyclonal biotinylated goat, # BAF212, 1:250	R&DSystems, Wiesbaden
Iba-1, polyclonal rabbit**,** # 019-19741, 1:300	Wako, Neuss
pERK, monoclonal 1,2 phospho p44/42/MAPK, rabbit, # 4376S, 1:1000	Cell signaling Technology, Frankfurt am Main
pAkt, monoclonal 1,2 phospho-Akt , rabbit, # 3787S, 1:100	Cell signaling Technology, Frankfurt am Main
GFP, polyclonal rabbit, # SC-8334 (WB: 1:3000; IHC: 1:500)	Santa Cruz Inc. Santa Cruz, USA
FLAG, monoclonal mouse, # 200471, 1:2000	Stratagene, La Jolla, USA
GFAP, polyclonal rabbit, # Z0334, 1:500	Dako Deutschland GmbH, Hamburg
NeuN, monoclonal mouse # MAB377, 1:500	Chemicon Temecula, USA
synphilin-1, polyclonal rabbit, # S5946, 1:200	Sigma-Aldrich Chemie GmbH, Taufkirchen
synphilin-1, polyclonal rabbit, # 1007, 1:1000	provided by C. O'Farrell, Jacksonville, USA
TH, polyclonal rabbit, # AB152, 1:1000	Chemicon Temecula, USA
β-Actin, monoclonal mouse, # A-5441, 1:10000	Sigma-Aldrich Chemie GmbH, Taufkirchen

secondary antibodies	
HRP-sheep anti-mouse Ig, #NXA931, 1:10000	Amersham Pharmacia Biotech, USA
HRP-donkey anti-rabbit IgG, #NA934V, 1:10000	Amersham Pharmacia Biotech, USA
Alexa555 goat anti-rabbit IgG, (red), 1:1000	Invitrogen GmbH, Karlsruhe
Alexa555 goat anti-mouse IgG, (red), 1:1000	Invitrogen GmbH, Karlsruhe
Biotin goat anti-rabbit IgG, 1:200, # BA-1000	Vector Laboratories, Loerrach
Biotin horse anti-goat IgG, 1:200, # BA-9500	Vector Laboratories, Loerrach

4.9.9 Bacterial strains

SURE 2 super chemicompetent	Stratagene, La Jolla, USA
DH-5α maximum efficiency	Invitrogen GmbH, Karlsruhe

4.9.10 Cell lines

HEK293FT	Invitrogen GmbH, Karlsruhe

4.9.11 Mouse lines

C57Bl/6-J
C57Bl/6-J, *Ret* ^{lx}
C57Bl/6-J, *DAT-Cre*
C57Bl/6-J, *DAT/Ret* $^{lx/lx}$

4.9.12 Real time PCR primer

Name	**Sequence**
TH sense	5′-GGTATACGCCACGCTGAAGG-3′
TH antisense	5′-TAGCCACAGTACCGTTCCAGA-3′
GAPDH sense	5′-TGGCAAAGTGGAGATTGTTGCC-3′
GAPDH antisense	5′-AAGATGGTGATGGGCTTCCCG-3′

4.9.13 Vector maps

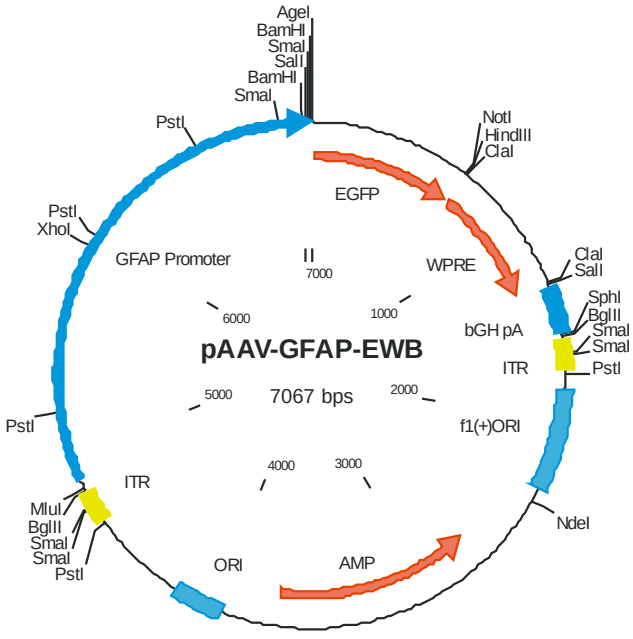

Fig. 4.1: pAAV-GFAP-EWB

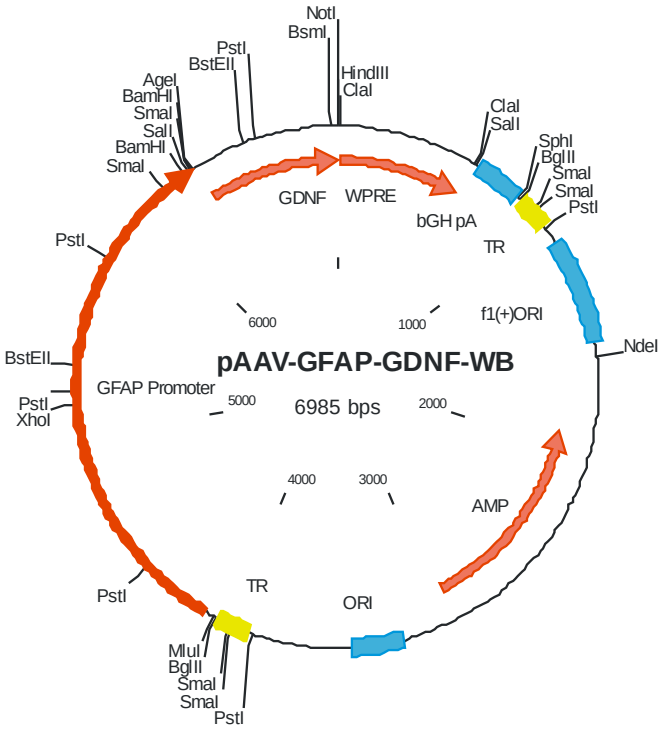

Fig: 4.2: pAAV-GFAP-GDNF-WB

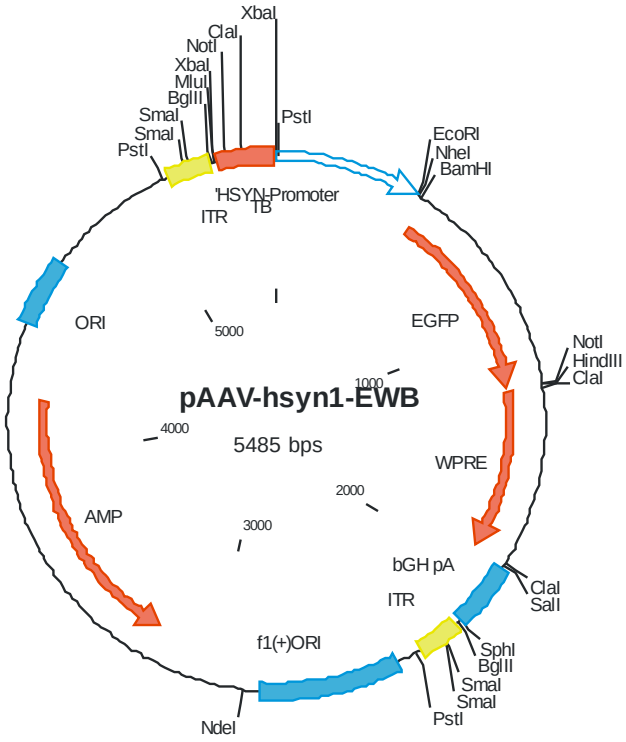

Fig. 4.3: pAAV-hsyn1-EWB

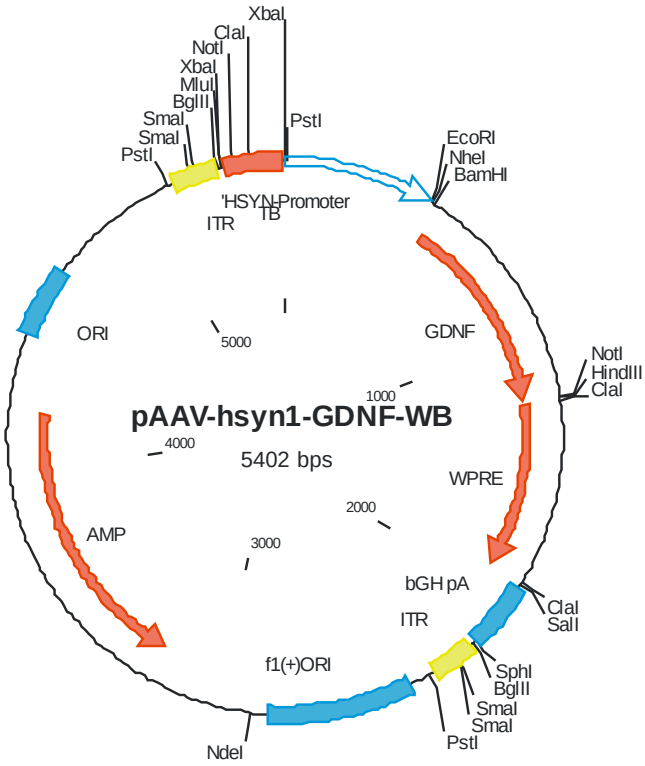

Fig. 4.4: pAAV-hsyn1-GDNF-WB

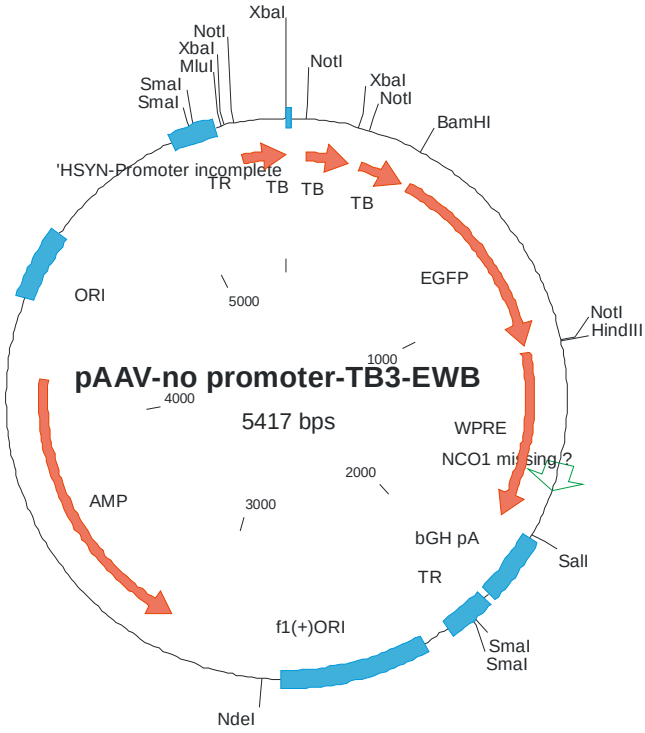

Fig. 4.5: pAAV-no promoter-TB3-EWB

5 Results

5.1 Astrocytic versus neuronal expression of GDNF

5.1.1 Properties of AAV-5 vectors driving transgene expression from GFAP or SYNapsin promoters

The injection of $2x10^8$ AAV-5 GFAP GFP and AAV-5 SYN GFP into the *striatum* of C57BL-6/J male mice resulted in a broad spatial distribution of transduced cells all-over the entire *striatum* and parts of the cortex two weeks and three months after the injection (Fig 5.1 and 5.2 A-B). The injection of $2x10^7$ transducing units resulted in a definite minor GFP expression per cell with less dense distribution of transduced cells two weeks and three months after injection (Fig 5.1 and 5.2 C-D). While AAV-5 GFAP GFP injection resulted in GFP expression exclusively in astrocytes (Fig. 5.1, 5.2 A and C, Fig. 5.3 A), AAV-5 SYN GFP injection resulted in neurons expressing the transgene (Fig. 5.1, 5.2 B and D, Fig. 5.3 B) for both viral titres.

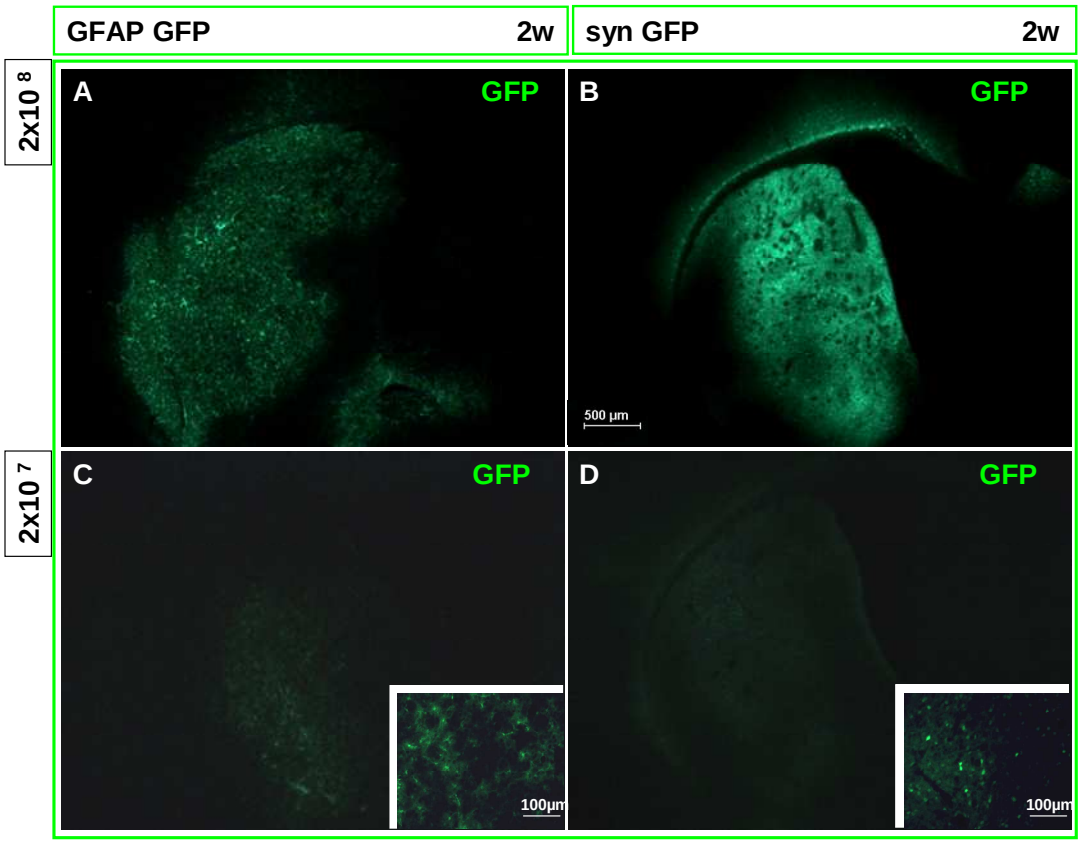

Fig. 5.1: Striatal GFP expression two weeks (2w) after AAV-5-mediated gene transfer. *Overview pictures of coronal sections showing striatal GFP expression two weeks after AAV-5 infection, expressing GFP under the control of the astrocyte-specific GFAP promoter (A and C) and under the control of the neuron-specific SYN promoter (B and D) for $2x10^8$ transducing units (A-B) and $2x10^7$ transducing units (C-D). Close-up of coronal sections for $2x10^7$ transducing units of AAV-5 GFAP GFP (C) and AAV-5 SYN GFP (D) –infected animals showing detailed GFP expression in the striatum.*

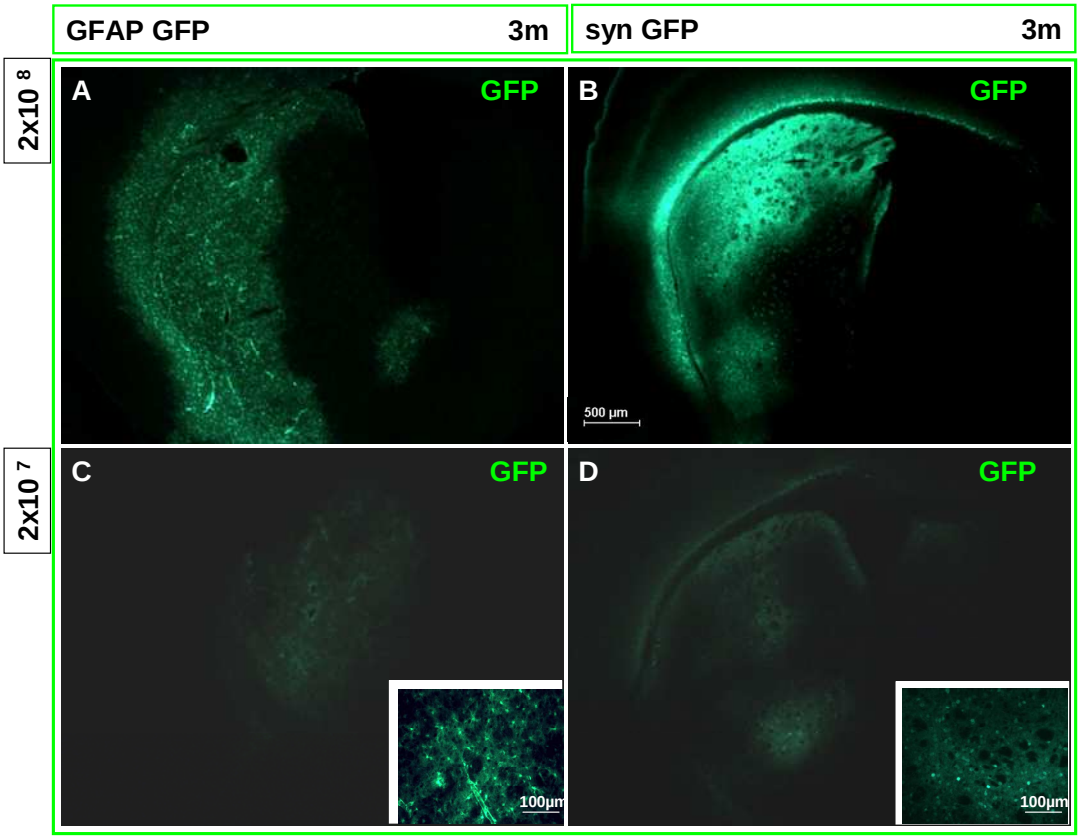

Fig. 5.2: Striatal GFP expression three months (3m) after AAV-5-mediated gene transfer. *Overview pictures of coronal sections showing striatal GFP expression three months after AAV-5 infection, expressing GFP under the control of the GFAP promoter (A and C) and under the control of the SYN promoter (B and D) for 2x10^8 (A-B) and 2x10^7 transducing units (C-D). Close-up of coronal sections for 2x10^7 transducing units of AAV-5 GFAP GFP (C) and AAV-5 SYN GFP (D) –infected animals showing detailed GFP expression in the striatum.*

The striatal injection of 2x10^8 AAV-5 GFAP GDNF and AAV-5 SYN GDNF resulted in a broad spatial distribution of GDNF throughout the entire *striatum* and parts of the cortex two weeks and three months after infection (Fig. 5.4 C-F). GDNF detection was based on ABC/DAB amplification. Striatal slices from AAV-5 GFAP GFP and AAV-5 SYN GFP-infected mice served as negative control (Fig. 5.4 A-B). The results in the SNpc are completely different, because the transport of GDNF from the *striatum* to the ipsilateral SNpc is more efficient after infection of neurons (Fig. 5.4 G-J). Furthermore, GDNF expressed by neurons was also detected in areas outside the *pars compacta*, e.g. the *pars reticulata* (Fig. 5.4 H and J).

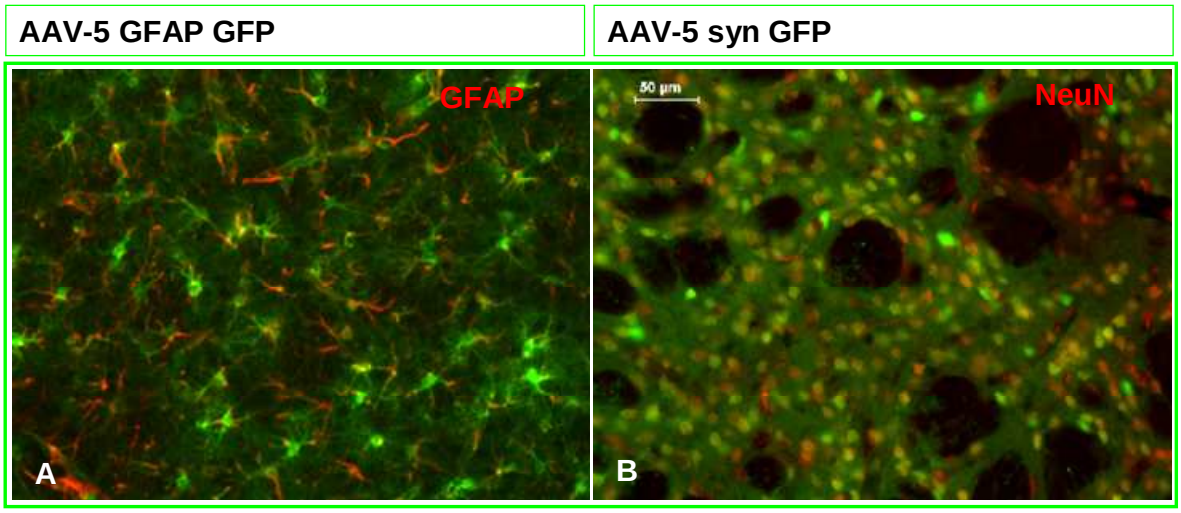

Fig. 5.3: Cell-specific GFP expression after AAV-5-mediated gene transfer (2x10^8 infectious particles, two weeks after infection). *Close-up of the striatum showing AAV-5 GFP expression exclusively in astrocytes defined by colocalization with GFAP (astrocytic marker, red) -positive cells if controlled by the GFAP promoter (A) and exclusively in neurons defined by colocalization with NeuN (neuronal marker, red) -positive cells if controlled by the SYN promoter (B).*

The injection of 2x10^7 transducing units resulted in a definite minor striatal distribution of GDNF two weeks and three months after infection for both viruses (Fig 5.5 A-D). The situation in the SNpc is comparable to the high titre dosage, with the difference that, due to a minor signal, GDNF is not longer detectable for animals infected with AAV-5 GFAP GDNF (Fig. 5.5 E-H).

In order to quantify the GDNF amount in the injected and non-injected *striatum* (CPu) and SN, a GDNF ELISA (Promega) was used. Samples were prepared two weeks after viral injection and have been diluted within a range of 1:5 up to 1:10.000 to find individually optimal ODs. Samples were tested in duplicates in three independent ELISA measurements. The ELISA revealed only small differences in the ipsilateral striatal amount of GDNF upon injection of high viral titres and no differences upon injection of low ones when comparing astrocytic and neuronal produced GDNF (Fig. 5.6). After neuronal infection (2x10^8 AAV-5 SYN GDNF) more GDNF is transported to the ipsilateral SN (13% of the striatal amount) in comparison to the infection of astrocytes with 2x10^8 AAV-5 GFAP GDNF (1.3% of the striatal amount). These results are in line with the immunohistology data for GDNF (Fig. 5.4). Although not detectable in immunohistology, the amount of GDNF was not only increased in the ipsilateral *striatum* and SN but also in the contralateral structures.

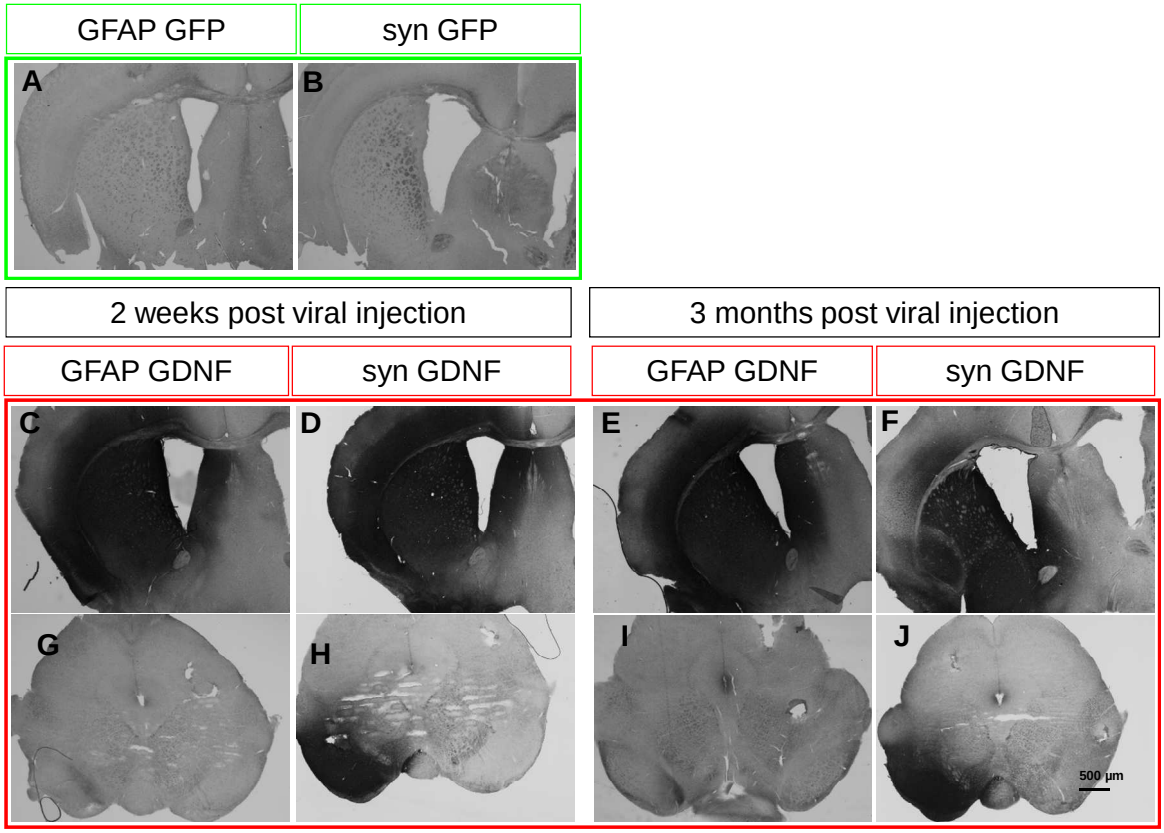

Fig. 5.4: GDNF overexpression in the striatum and SNpc of infected mice for 2x10^8 transducing units. *Overview pictures of coronal sections showing GDNF overexpression two weeks (A-D and G-H) and three months (E-F and I-J) after unilateral AAV-5 GFAP GFP or AAV-5 SYN GFP (A-B) and AAV-5 GFAP GDNF or AAV-5 SYN GDNF (C, E, G, I; D, F, H J) infection. Pictures are shown with the injected striatum (A-F) and SNpc (G-J) on the left.*

The 8-fold higher amount of GDNF found in the contralateral *striatum* after infection with AAV-5 SYN GDNF (25-fold increase over baseline) compared to infection with AAV-5 GFAP GDNF (3-fold increase over baseline) shows a tendency to a more efficient transport to the contralateral *striatum* after neuronal infection. Such a tendency could not be detected for the contralateral SN (Fig. 5.6).

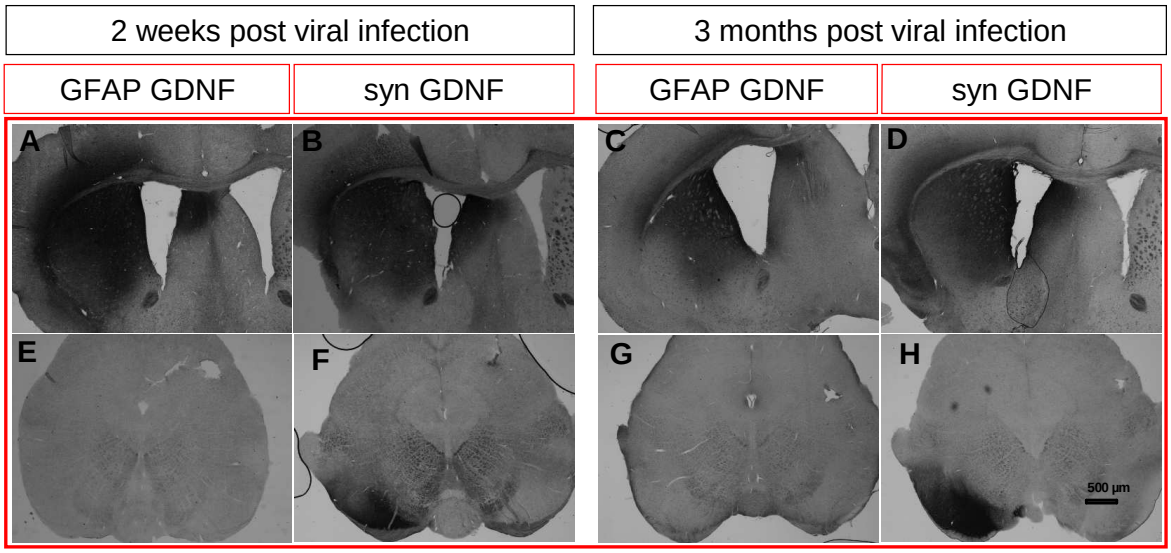

Fig. 5.5: GDNF overexpression in the striatum and SNpc of infected mice for 2x10^7 transducing units. *Overview pictures of coronal sections showing GDNF overexpression two weeks (A-B and E-F) and three months (C-D and G-H) after unilateral AAV-5 GFAP GDNF or AAV-5 SYN GDNF (A, C, E, G; B, D, F, H) infection. Pictures are shown with the injected striatum (A-D) and SNpc (E-H) on the left.*

After infection with 2x10^7 transducing units astrocytes and neurons produce the same amount of GDNF ($p > 0.05$) but both produced considerable less amount of GDNF in comparison to the high titre dosage in the ipsilateral *striatum* (Fig. 5.6). Again, after neuronal infection a higher amount of GDNF is transported to the ipsilateral SN (39% of the striatal amount) in comparison to the infection of astrocytes (3.6% of the striatal amount) ($p < 0.001$) (Fig. 5.6). These results are again in line with the immunohistology data for GDNF (Fig. 5.5).
Although having used 10-fold less virus, the percentage of GDNF transported from the *striatum* to the contralateral SN is higher for both neuronal and astrocytic GDNF production (Fig. 5.6). In contrast to the high titre dosage, the contralateral *striatum* or SN did not show increased GDNF amounts (Fig. 5.6).
Comparing the ELISA results to the immunohistology for GDNF, in this study the detection limit for GDNF on slices can be defined at ~ 1100pg GDNF/mg tissue (Fig. 5.4, 5.5, 5.6).

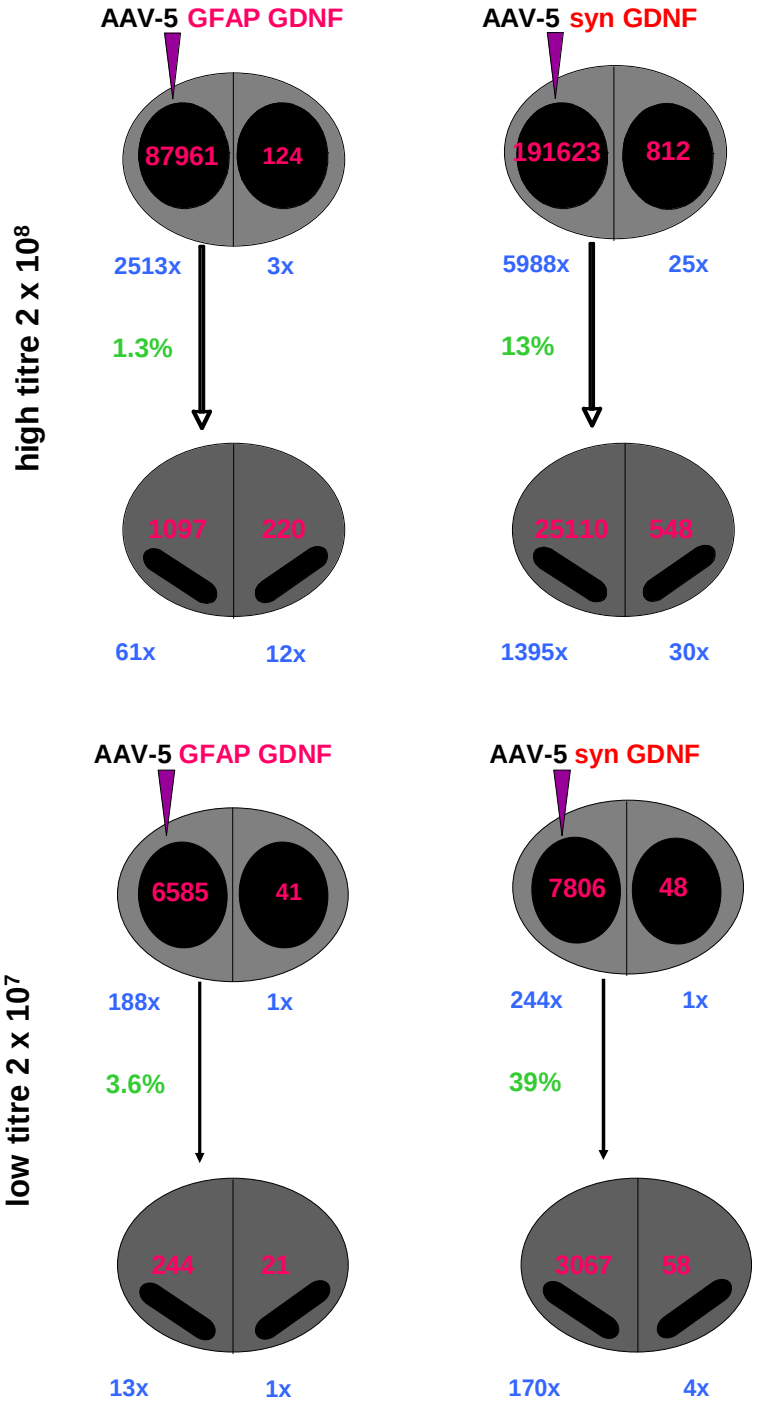

Fig. 5.6: Quantification of GDNF in the striatum (CPu) and SN in mice after high and low titre infection by ELISA. *Red values represent mean pg GDNF/mg CPu or SN, blue values mark the fold-increase to background levels and green values demonstrate the fraction of the GDNF amount in the injected site, which is transported to the ipsilateral SN. Pictures on the top of each titre condition demonstrate the CPu and the SN on the bottom. Animals were sacrificed two weeks after unilateral infection (n=3).*

(i) The AAV-5 serotype efficiently transduces both neurons and astrocytes, but transcriptional control is able to completely restrict transgene expression to either neurons or astrocytes using $2x10^8$ and $2x10^7$ transducing units.
(ii) Neuronal and astrocytic expression of GFP and GDNF in the striatum is detectable without differences regarding distribution or expression level for both time points but transport of GDNF to the ipsilateral SN is much more efficient if GDNF is produced by neurons.
(iii) In addition to the transport of GDNF from the ipsilateral striatum to the ipsilateral SNpc, also other tracks from the ipsilateral to the contralateral striatum and SN or from the SN via anterograd transport to the striatum might also be in use in the presence of high GDNF amounts.

5.1.2 Tissue reactions to astrocytic or neuronal transgene expression

All stereotaxic injections provoked a glial response as evaluated by up-regulation of the astrocytic marker GFAP and detected by immunohistochemistry. Interestingly, this is also evident – though to a lesser extent after sodium chloride or empty AAV-5 (expressing no transgene at all) injection (data not shown).

At two weeks after injection of $2x10^8$ transducing units AAV-5 GFAP GFP and AAV-5 SYN GFP an astrogliosis in the *striatum* could be detected for both of them (Fig. 5.7 A-B). For the low titre dosage ($2x10^7$ transducing units) the GFAP immunoreactivity was reduced (Fig. 5.7 C-D). The two weeks time point after AAV-5 GFAP GDNF and AAV-5 SYN GDNF injection shows the same pattern but only a slight reduction of astrogliosis in the presence of low titre dosages (Fig. 5.8).

At three months after stereotaxic injection astrogliosis has clearly faded for both AAV-5 GFAP GFP and AAV-5 SYN GFP for the high titre dosage and disappeared for the low titre dosage (Fig. 5.9). The three months time point after AAV-5 GFAP GDNF and AAV-5 SYN GDNF injection shows the same but more differentiated pattern (Fig. 5.10).

Evaluating microglial response to cell type-specific transgene expression, astrocytic GFP and GDNF expression at the high titre dosage were the only paradigms that provoked a mild increase in ionized calcium binding adaptor molecule-1 (Iba-1) immunoreactivity (Fig. 5.11 A, 5.12 A). This increase in Iba-1 immunoreactivity was no longer detected at three months after stereotaxic injection (data not shown). Injection of AAV-5 SYN GFP and AAV-5 SYN GDNF for both viral titres (Fig. 5.11 B and D, 5.12 B and D) and injection of AAV-5 GFAP GFP and AAV-5 GFAP GDNF only for the low titre dosage (Fig. 5.11 C, 5.12 C) did not result in any activation of microglial markers.

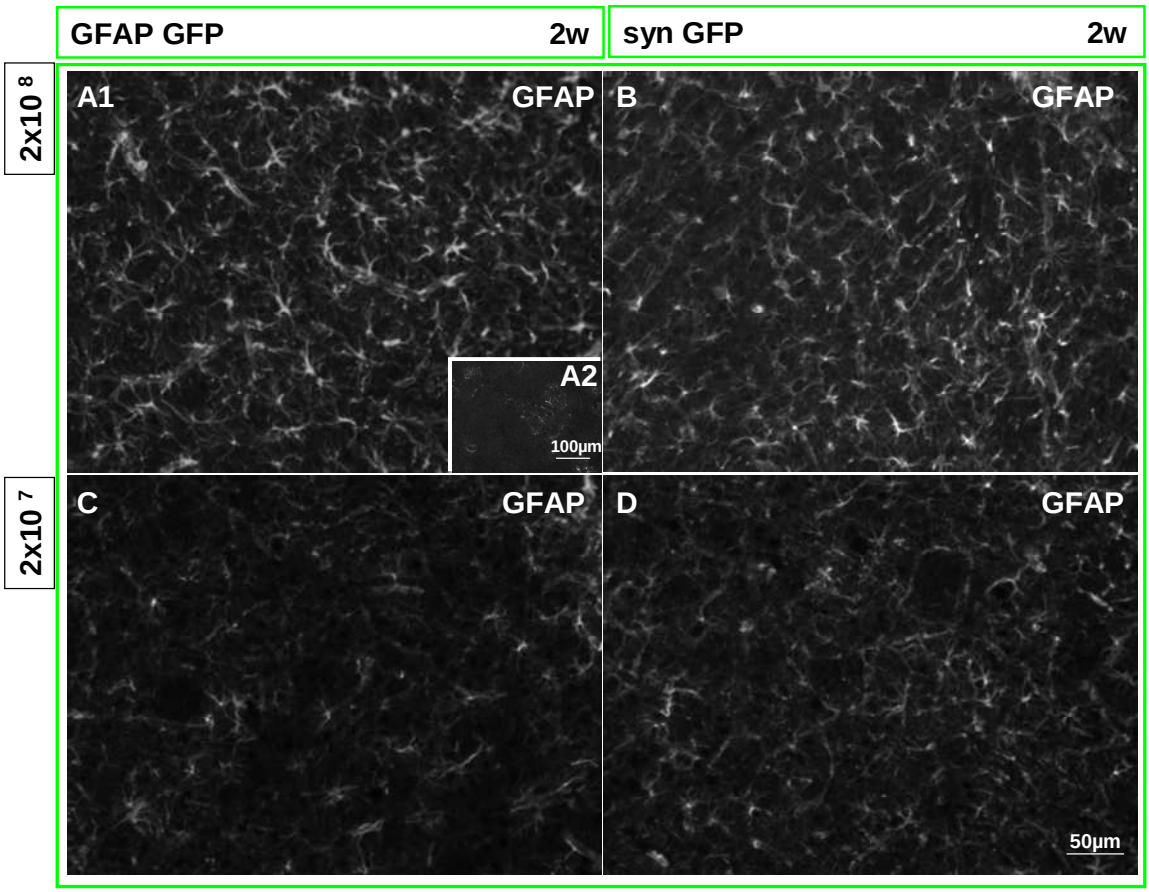

Fig. 5.7: Reaction of astrocytes two weeks (2w) after unilateral injection of AAV-5 GFP in the striatum. *Close-up of coronal sections stained for the astrocytic marker GFAP, showing the same reaction of astrocytes after stereotaxic injection of AAV-5 GFAP GFP (A) compared to AAV-5 SYN GFP (B) for the high titre dosage (2x10^8 transducing units). For the low titre dosage (2x10^7 transducing units) the GFAP immunoreactivity was reduced for both AAV-5 GFAP GFP (C) and AAV-5 SYN GFP (D) -infected animals. A2 shows GFAP immunoreactivity of an uninjected control slide.*

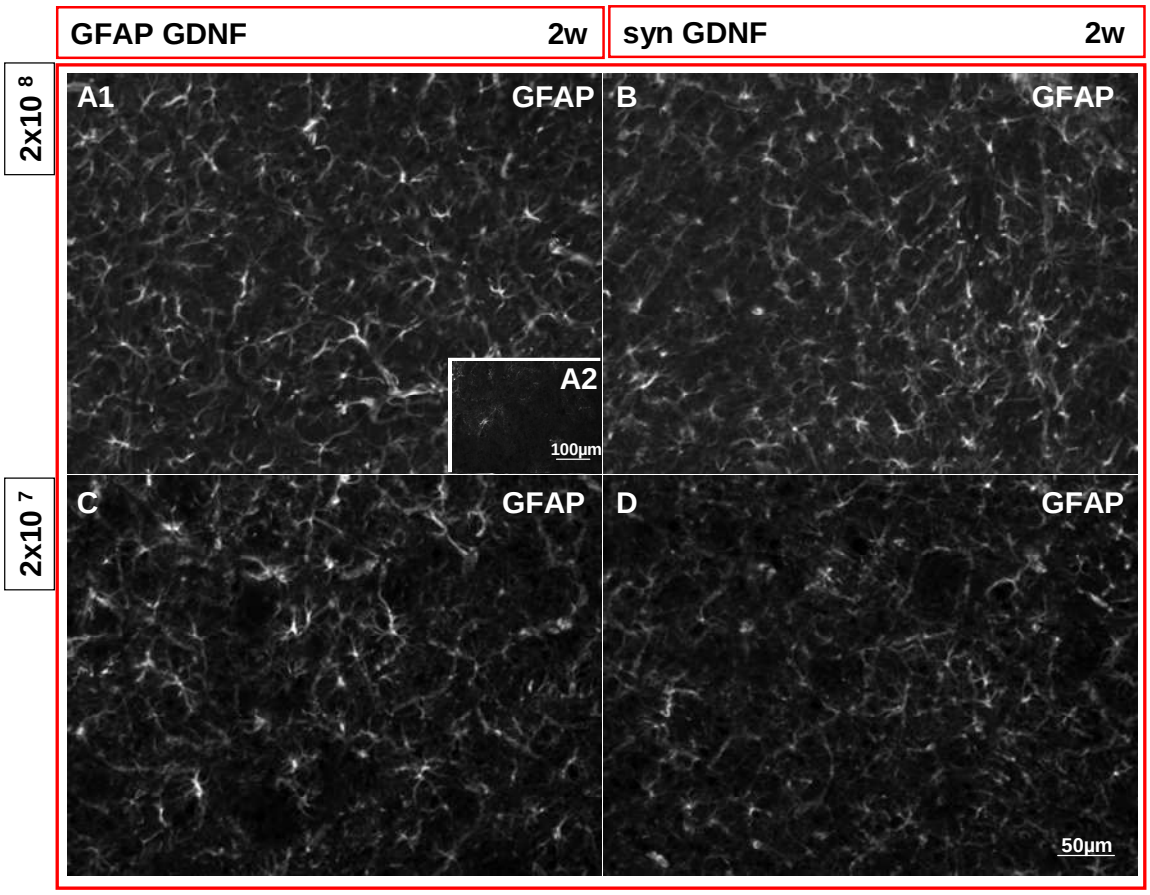

Fig. 5.8: Reaction of astrocytes two weeks (2w) after unilateral injection of AAV-5 GDNF in the striatum. *Close-up of coronal sections stained for the astrocytic marker GFAP, showing the same reaction of astrocytes after stereotaxic injection of AAV-5 GFAP GDNF (A) compared to AAV-5 SYN GDNF (B) for the high titre dosage (2x10^{8} transducing units). For the low titre dosage (2x10^{7} transducing units) the GFAP reactivity was only slightly reduced after both AAV-5 GFAP GDNF (C) and AAV-5 SYN GDNF (D) infection. A2 shows GFAP immunoreactivity of an uninjected control slide.*

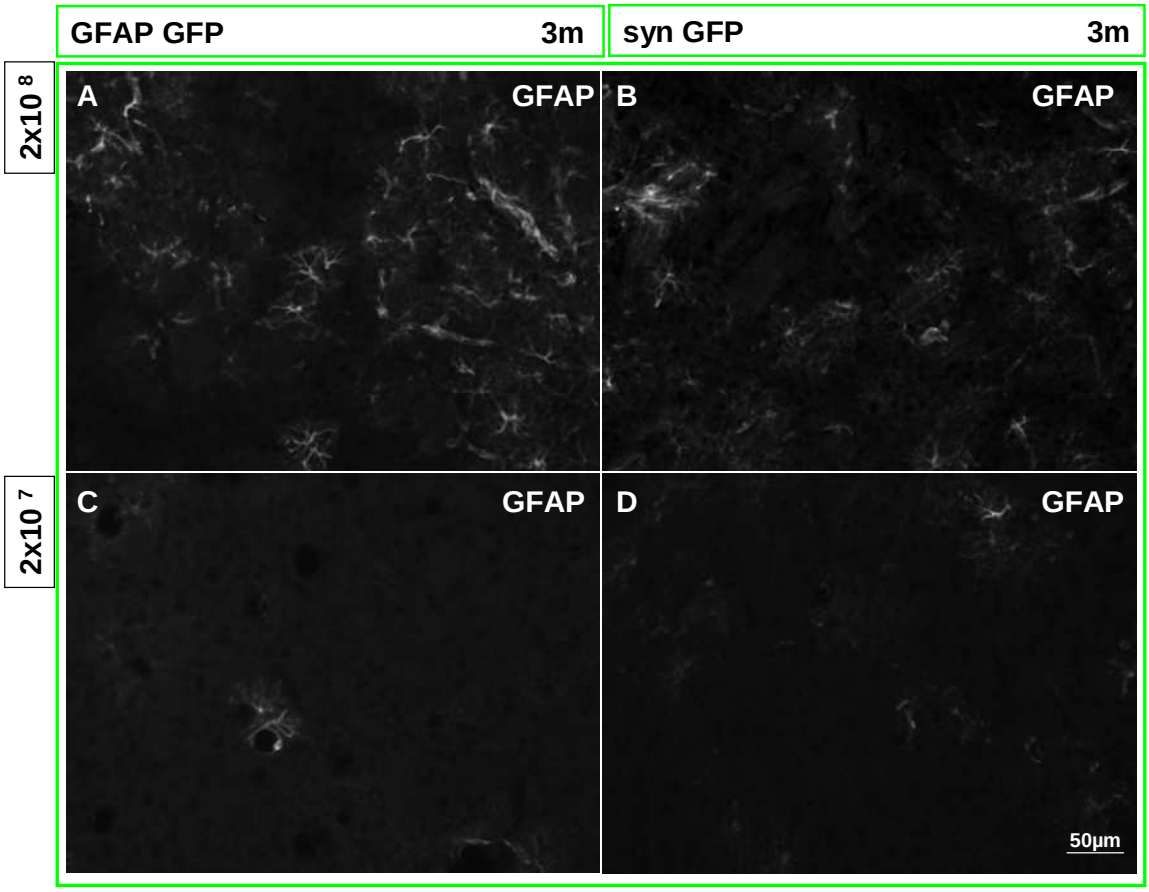

Fig. 5.9: Reaction of astrocytes three months (3m) after unilateral injection of AAV-5 GFP in the striatum. *Close-up of coronal sections stained for the astrocytic marker GFAP, showing a mild reaction of astrocytes three months after stereotaxic injection of both AAV-5 GFAP GFP (A) and AAV-5 SYN GFP-infected animals (B) for the high titre dosage (2x10^8 transducing units). For the low titre dosage (2x10^7 transducing units) an increased GFAP reactivity in comparison to negative controls was not detected in either AAV-5 GFAP GFP (C) or AAV-5 SYN GFP (D) -infected animals.*

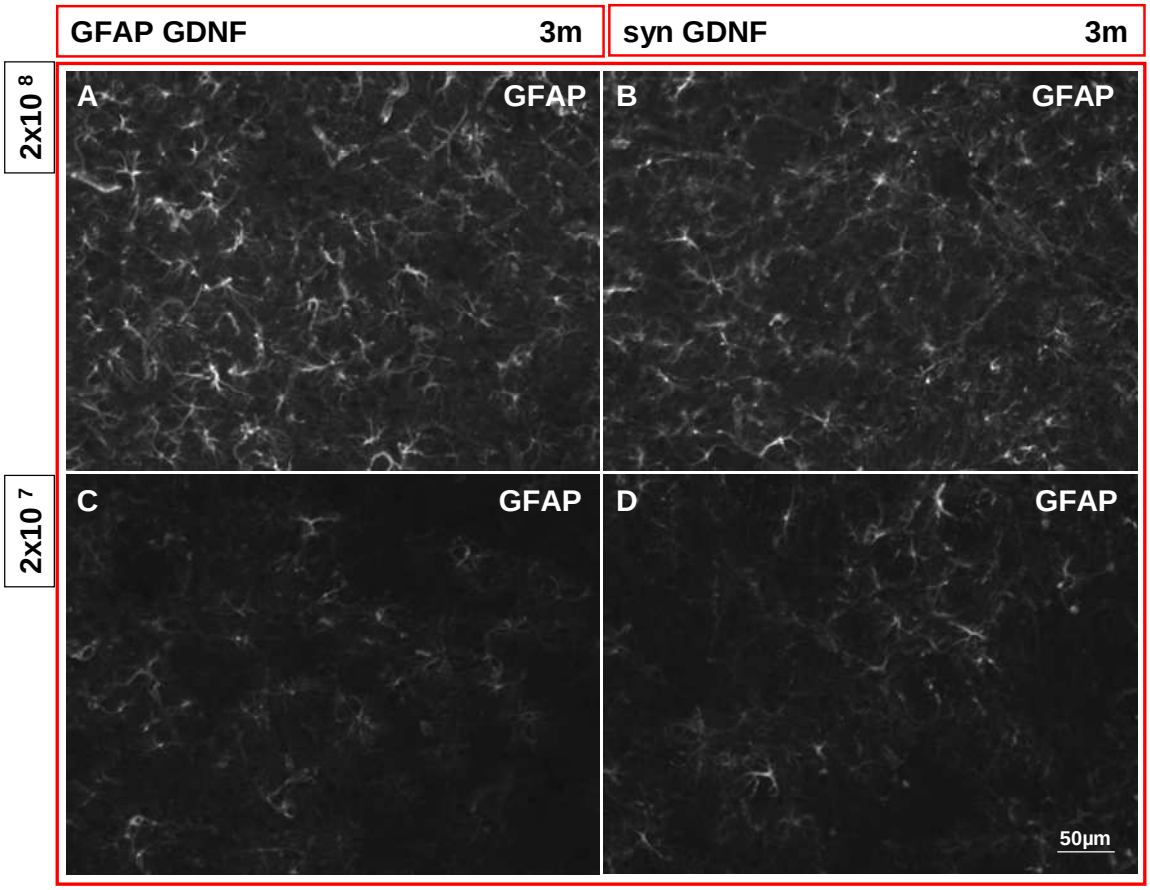

Fig. 5.10: Reaction of astrocytes three months (3m) after unilateral injection of AAV-5 GFP in the striatum. *Close-up of coronal sections stained for the astrocytic marker GFAP, showing a reaction of astrocytes three months after stereotaxic injection of both AAV-5 GFAP GFP (A) and AAV-5 SYN GFP-infected animals (B) for the high titre dosage (2x10^{8} transducing units). For the low titre dosage (2x10^{7} transducing units) the GFAP immunoreactivity is clearly reduced but still detectable.*

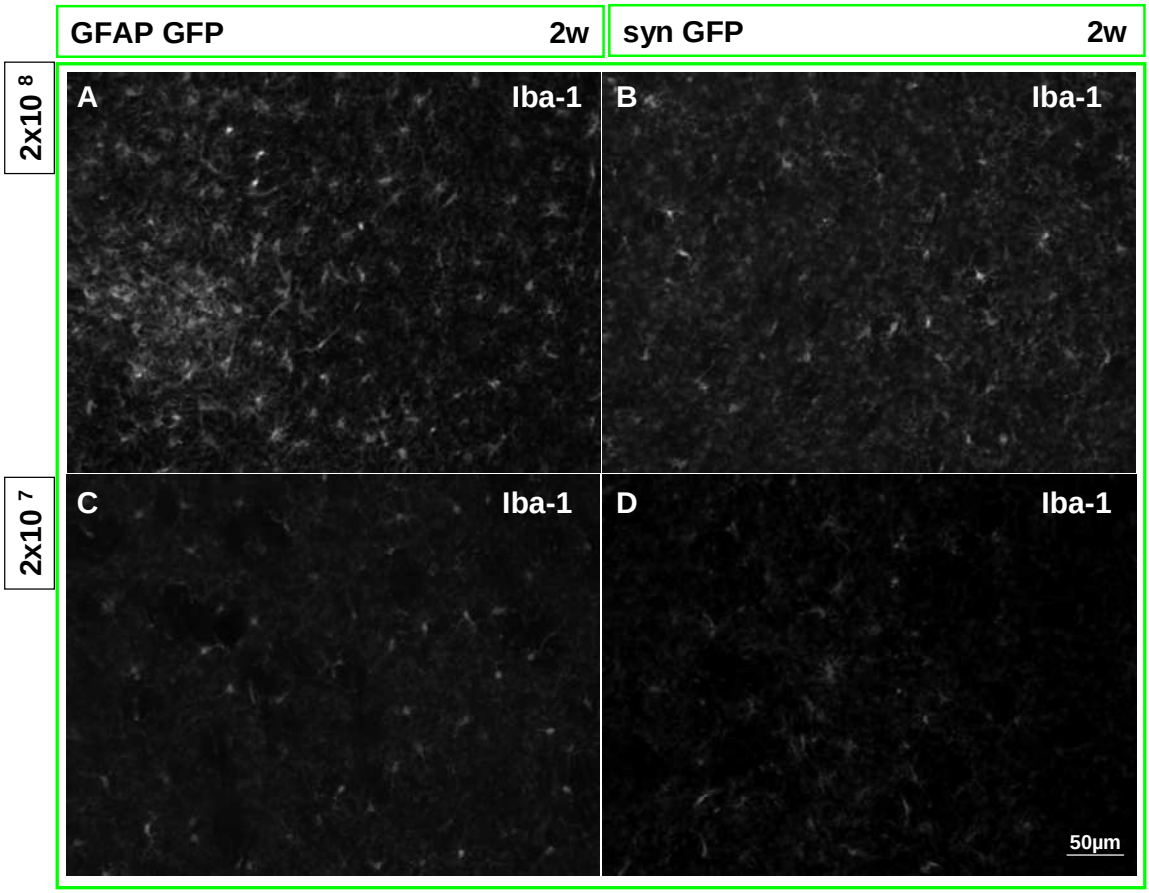

Fig. 5.11: Reaction of microglia two weeks (2w) after unilateral injection of AAV-5 GFP in the striatum. *Close-up of coronal sections stained for the microglial marker Iba-1, showing a very mild reaction of microglia only after stereotaxic injection of AAV-5 GFAP GFP (A) for the high titre dosage (2x10^8 transducing units). For AAV-5 SYN GFP (B) at the high titre dosage and both AAV-5 GFAP GFP (C) and AAV-5 SYN GFP (D) at the low titre dosage of 2x10^7 transducing units no increased microglial immunoreactivity was detected.*

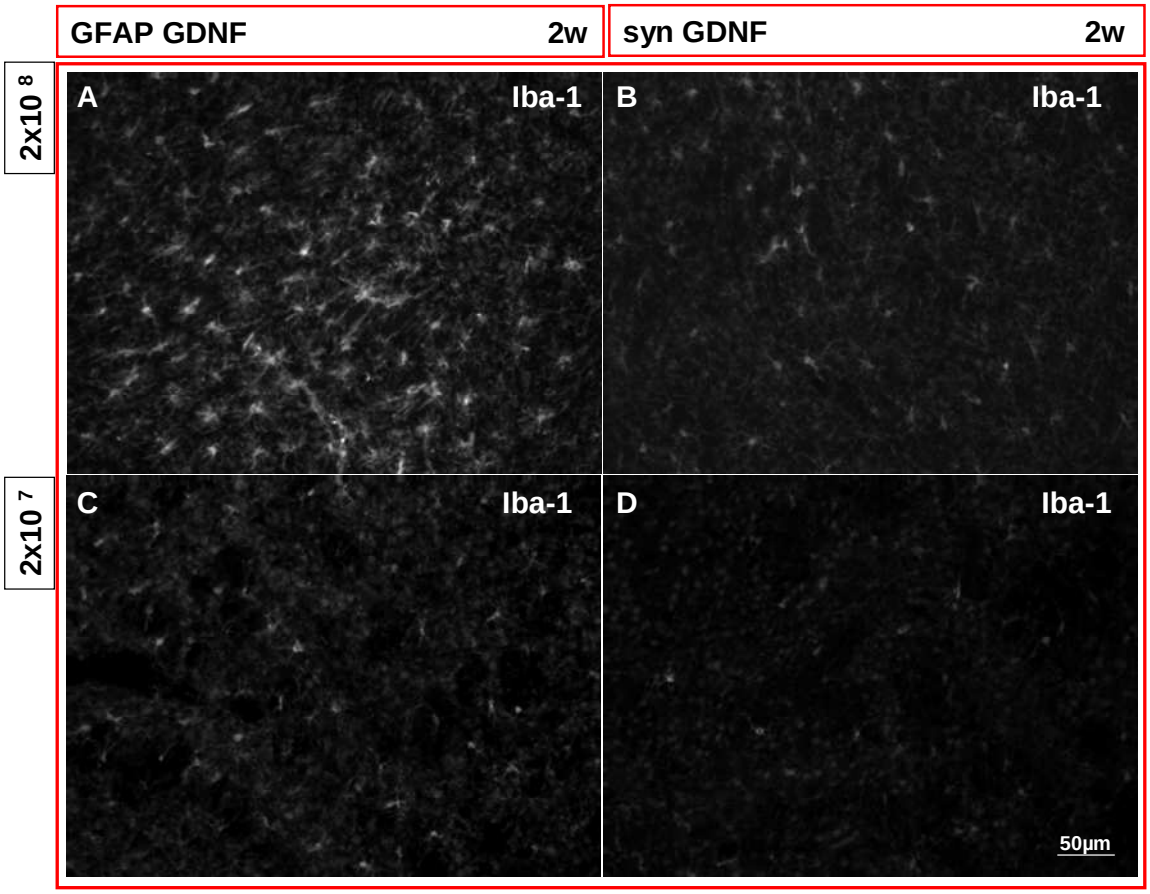

Fig. 5.12: Reaction of microglia two weeks (2w) after unilateral injection of AAV-5 GDNF in the striatum. *Close-up of coronal sections stained for the microglial marker Iba-1, showing a reaction of microglia only after stereotaxic injection of AAV-5 GFAP GDNF (A) for the high titre dosage (2x10^{8} transducing units). For AAV-5 SYN GDNF (B) at high titre dosage and both AAV-5 GFAP GDNF (C) and AAV-5 SYN GDNF (D) at low titre dosage of 2x10^{7} transducing units no increased microglial immunoreactivity was detected.*

(i) Stereotaxic injection itself induces astrogliosis; the presence of an AAV-5 and the overexpression of the transgenes GFP and GDNF potentiate it.

(ii) The same level of astrogliosis was detected for astrocytic and neuronal transgene expression at high and low titre dosages.

(iii) The recovery from astrogliosis argues against chronic activation of astrocytes.

(iv) Evaluating microglial response to cell type-specific transgene expression, astrocytic GFP and GDNF expression at high titre dosage were the only paradigms to provoke a mild and short-term microgliosis.

5.1.3 GDNF-mediated neuroprotection in the subchronic MPTP model of PD

GDNF or GFP expressing AAV-5 vectors were unilaterally injected into the *striatum* of two-three month old Bl-6/J male wildtype mice and after two weeks MPTP was administered subchronically. Unilateral virus injection had the advantage of using the contralateral side as internal control for each animal. GDNF-mediated neuroprotection against MPTP toxicity was assessed by several means either two weeks or three months after MPTP applications, making it possible to distinguish between acute and regenerative effects of GDNF: stereological quantification of nigral DA neurons, quantitative assessment of TH positive striatal fibre density, determination of striatal levels of DA and its metabolites DOPAC and HVA and functional analysis of motor control by rotarod. The experiments were performed using $2x10^8$ and $2x10^7$ transducing units as viral titres in order to find out, which cell type-specific GDNF expression is leads to the most protective and regenerative effects: astrocytic (AAV-5 GFAP GDNF) or neuronal GDNF (AAV-5 GFAP GDNF) expression.

5.1.3.1 Stereological quantification of nigral DA neurons

Stereological quantification was accomplished by counting both TH and Nissl-positive cells in the SNpc. Nissl stain served as a TH-independent neuronal marker. The counting revealed that expression of GFP in either astrocytes or neurons had no effect in comparison to the contralateral SN for the high titre dosage, true for both time points (Fig. 5.13 B-E and Fig. 5.14 A,B). At two weeks after MPTP administration neuronal expression of GDNF resulted in a moderate but significant neuroprotection against MPTP toxicity in both the ipsilateral and the contralateral SNpc (Fig. 5.13 G and Fig. 5.14 A).

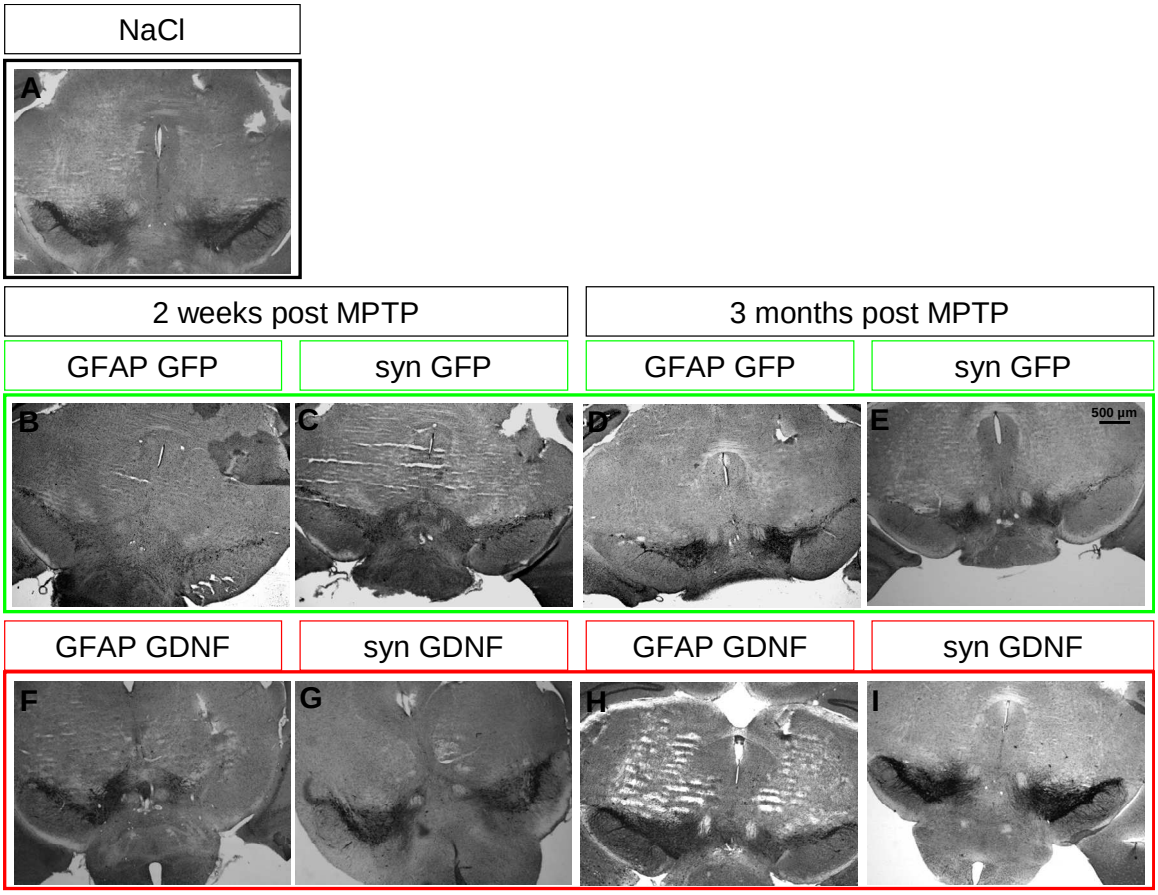

Fig. 5.13: DA SNpc neurons two weeks and three months after MPTP treatment for 2x10^8 transducing units. *Overviews of coronal sections stained for DA neurons of the SNpc by TH/DAB and Nissl. The MPTP-induced loss of SNpc neurons in the AAV-5 GFAP GFP and AAV-5 SYN GFP-injected control groups (B-E) can be prevented unilaterally in the AAV-5 GFAP GDNF (F, H) and bilaterally in the AAV-5 SYN GDNF-treated animals (G, I) for both timepoints. Animals injected intraperitoneally with sodium chloride (NaCl) served as control (A) for the MPTP-treated animals (B, I). left: virus-injected side; right: control side*

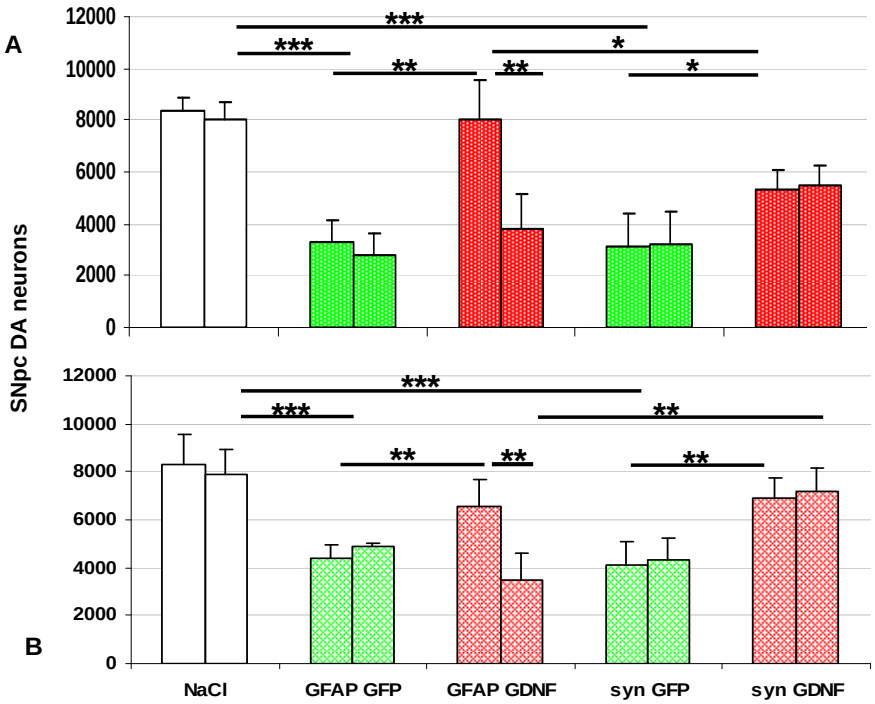

Fig. 5.14: Stereological counts for TH and Nissl-positive cells in the SNpc two weeks (A) and three months (B) after MPTP treatment for 2x10^8 transducing units. *Animals injected intraperitoneally with sodium chloride (NaCl) served as control (A) for the MPTP-treated animals. Bars represent mean ± SD; n = 4-5 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; left bar: virus-injected side; right bar: control side*

In contrast, astrocytic GDNF expression presented a complete preservation of DA SNpc neurons in the ipsilateral side, but no protection in the contralateral SNpc (Fig. 5.13 F and Fig. 5.14 A).

At three months after MPTP administration and neuronal GDNF expression now almost pre-lesion numbers of SNpc DA neurons in both brain hemispheres were counted (Fig. 5.13 I and Fig. 5.14 B). Although this increase in cell numbers showed only a tendency to significance, it indicates a certain recovery from MPTP toxicity – probably due to a short-term downregulation of TH in a fraction of DA neurons of the SNpc. Again, astrocytic GDNF expression exclusively resulted in an almost complete ipsilateral protection of DA neurons (Fig. 5.13 H and Fig. 5.14 B).

Neither did stereology of nigral DA neurons show any effect compared to the contralateral control side for the low titre dosage and both time points for AAV-5 GFAP GFP and AAV-5 SYN GFP treated animals (Fig. 5.15 B-E and Fig. 5.16 A, B). Two weeks after MPTP administration neuronal and astrocytic expression of GDNF resulted in an almost complete ipsilateral, but in no protection in the contralateral SNpc (Fig. 5.15 F, G and Fig. 5.16 A).

Three months after MPTP administration neuronal and astrocytic expression of GDNF still protects the ipsilateral SNpc (Fig. 5.15 H and Fig. 5.16 B). In AAV-5 SYN GDNF treated animals an additional but just rudimental protection of the contralateral SNpc was detected (Fig. 5.15 I and Fig. 5.16 B). Correlating the stereology results to the GDNF ELISA data, 244pg GDNF/mg SNpc are enough to be long-term protective to the ipsilateral injection side, whereas 220pg GDNF/mg SNpc are not sufficient to protect the contralateral SNpc (Fig. 5.6, 5.14 and 5.16). Thus, other factors in addition to the GDNF amount itself, can possibly make a crucial difference. One of these factors might be the anatomical position and connection to the injected tissue, resulting in alterations of signalling pathways and cellular responses.

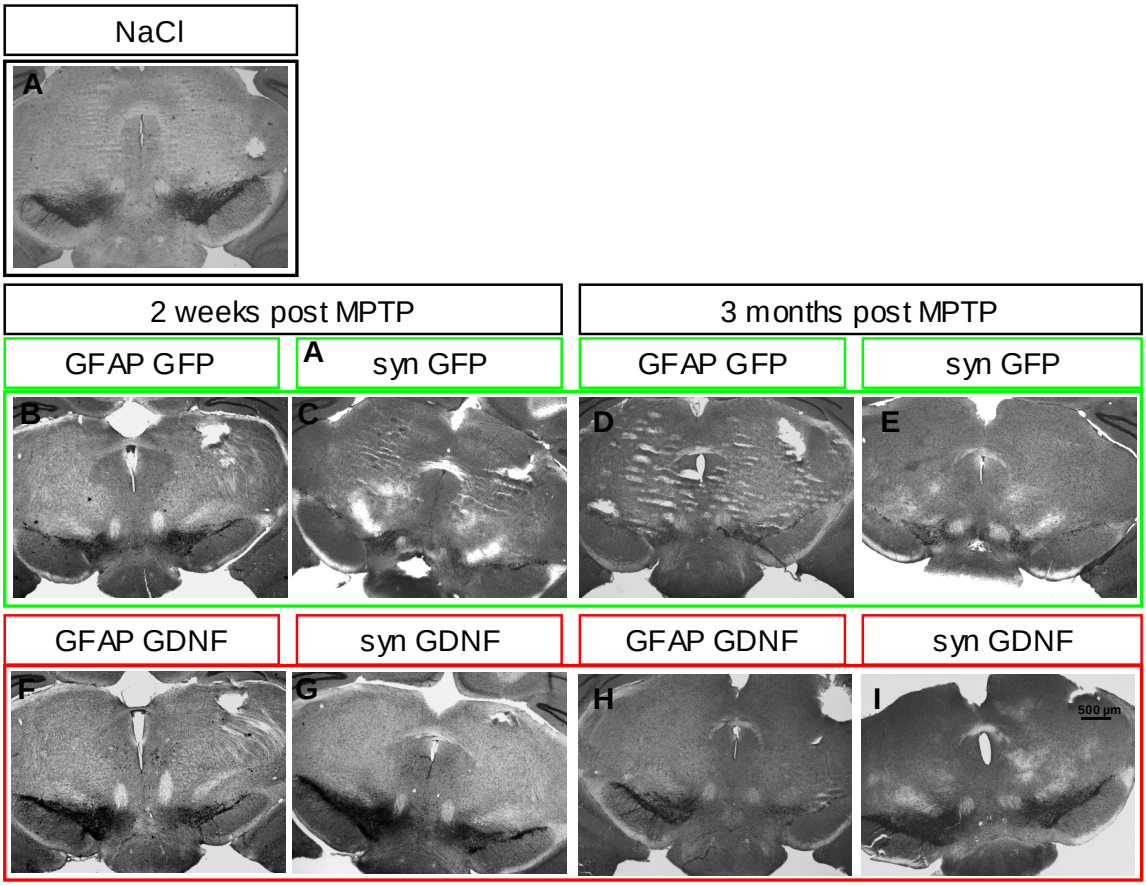

Fig. 5.15: DA SNpc neurons two weeks and three months after MPTP treatment for 2x10^7 transducing units. *Overview of coronal sections stained for DA neurons of the SNpc by TH/DAB and Nissl. The MPTP-induced loss of SNpc neurons in the AAV-5 GFAP GFP and AAV-5 SYN GFP-injected control groups (B-E) can be prevented unilaterally in the AAV-5 GFAP GDNF-treated animals for both time points (F, H) and in the AAV-5 SYN GDNF-treated animals unilaterally for the two weeks (G) and rudimental bilaterally for the three months time point (I). Animals injected intraperitoneally with sodium chloride (NaCl) served as control (A) for the MPTP-treated animals (B, I). left: virus-injected side; right: control side*

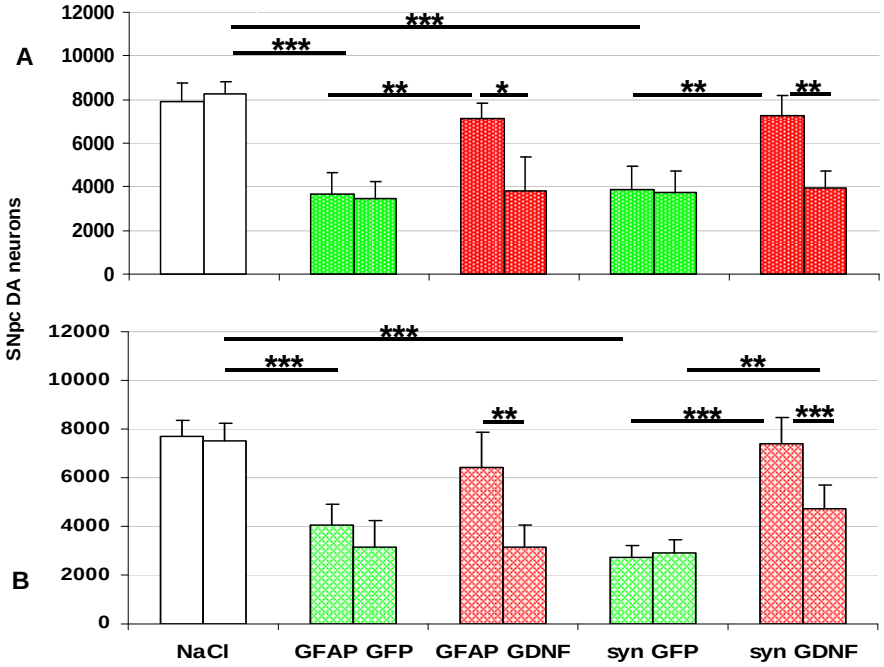

Fig. 5.16:* Stereological counts for TH and Nissl-positive cells in the SNpc two weeks (A) and three months (B) after MPTP treatment for $2x10^7$ transducing units.** *Animals injected intraperitoneally with sodium chloride (NaCl) served as control for the MPTP-treated animals. Bars represent mean ± SD; n = 4-5 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p< 0.001; left bar: virus-injected side; right bar: control side*

5.1.3.2 Assessment of striatal fibre density

A rapidly progressive degeneration of DA nigrostriatal projections, affecting about 80% in the *striatum*, was detected by TH/DAB immunoreactivity two weeks after MPTP administration (Fig. 5.17 B-E and Fig. 5.19 A). The quantification of the striatal fibre density revealed that GFP expression in either astrocytes or neurons had no effect compared to the contralateral side regarding both time points (Fig. 5.17 B-E; Fig. 5.18 B-E and Fig. 5.19 A, B). In congruence with the stereology it was found that two weeks after MPTP administration unilateral neuronal GDNF expression protected DA fibres in both the ipsi- and contralateral *striatum* (Fig. 5.17 H-I and Fig. 5.19 A). Again, astrocytic GDNF expression resulted in almost complete but ipsilaterally restricted protection of DA projections (Fig. 5.17 F-G and Fig. 5.19 A). Three months after MPTP administration the protective effect is still ipsilaterally limited (Fig. 5.18 F-G and Fig. 5.19 B). Animals, which have been treated with AAV-5 SYN GDNF show ipsi- and contralateral protection (Fig. 5.18 H-I and Fig. 5.19 B).

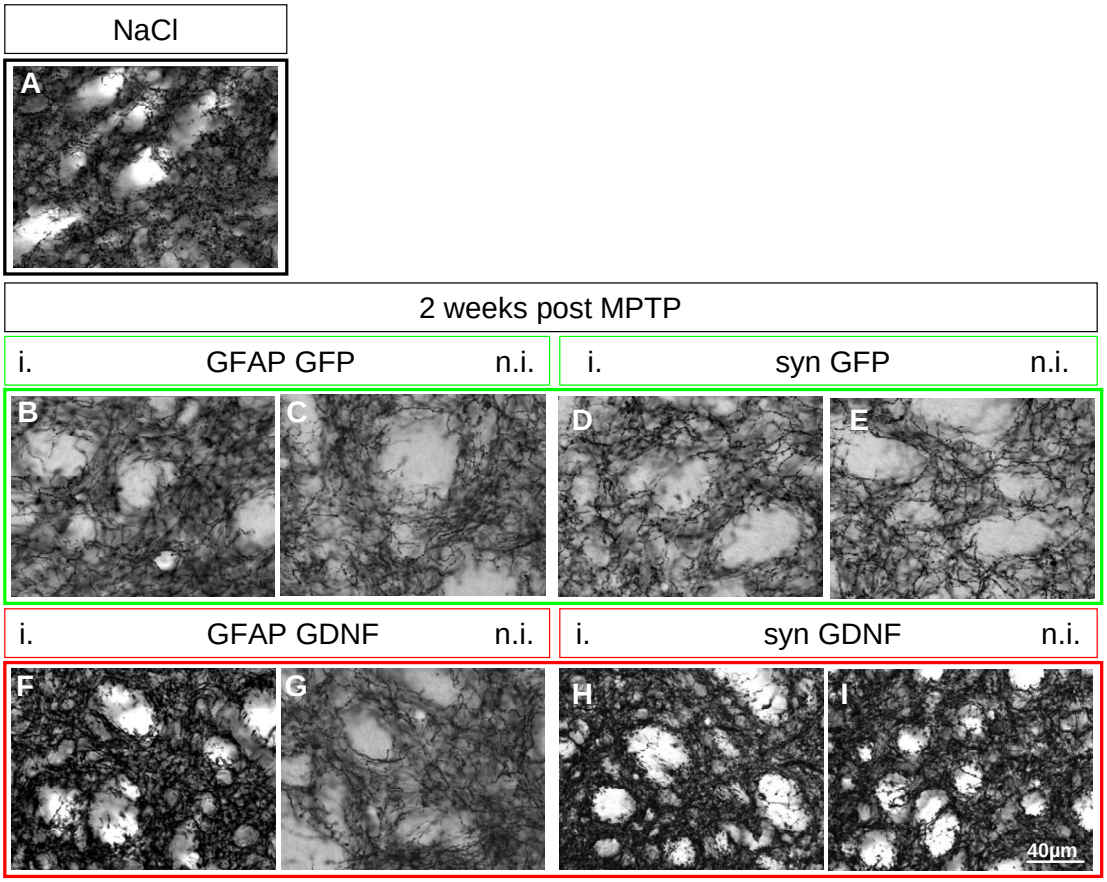

Fig. 5.17: TH fibre density in the striatum two weeks after MPTP treatment for 2x10^8 transducing units. *Close-up of coronal sections stained for DA fibres of the striatum by TH/DAB. The MPTP-induced loss of TH-positive fibres in the AAV-5 GFAP GFP and AAV-5 SYN GFP-injected control groups can be prevented unilaterally in the AAV-5 GFAP GDNF and bilaterally in the AAV-5 SYN GDNF-treated animals. Animals injected intraperitoneally with sodium chloride (NaCl) served as control (A) for the MPTP-treated animals (B, I) i.: virus-injected side n.i.: control side*

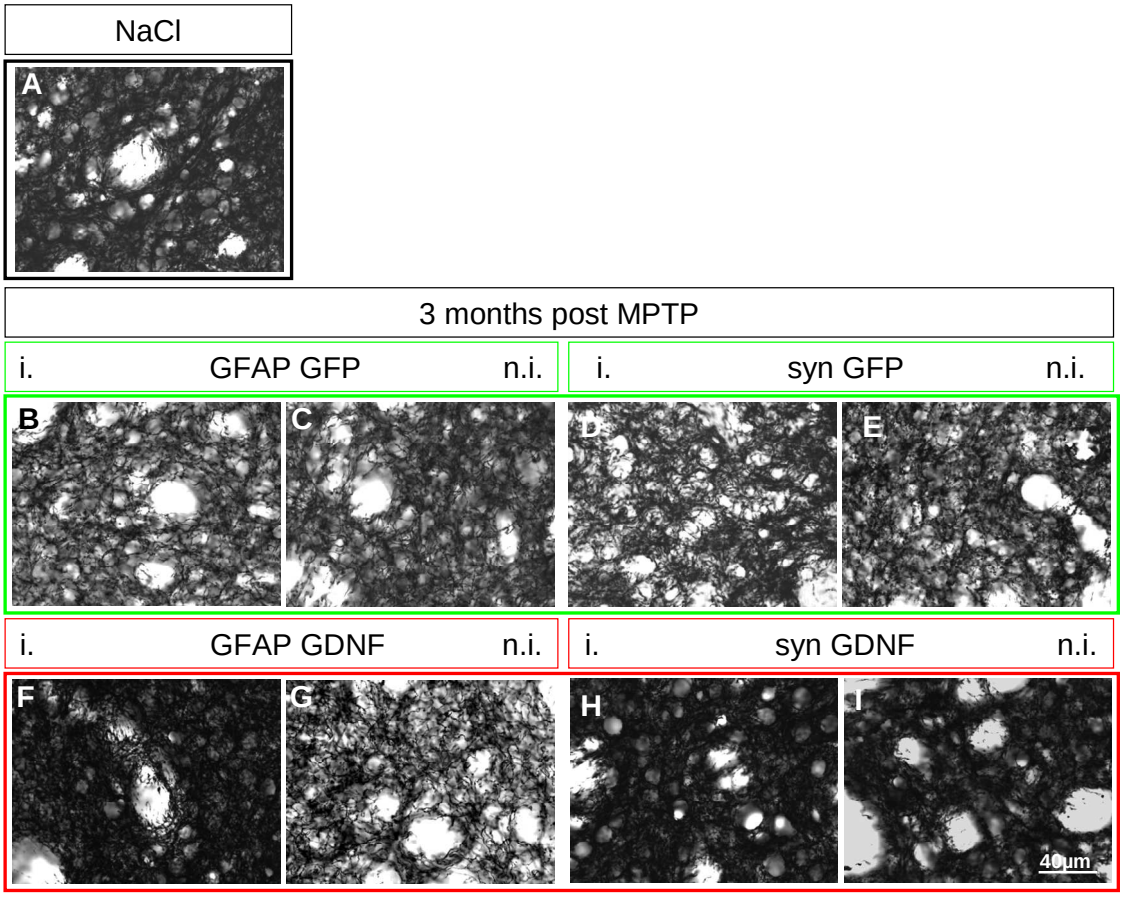

Fig. 5.18: TH fibre density in the striatum three months after MPTP treatment for 2x10^8 transducing units*. Close-up of coronal sections stained for DA fibres of the striatum by TH/DAB. The MPTP-induced loss of TH-positive fibres detected in the AAV-5 GFAP GFP and AAV-5 SYN GFP-injected control groups is prevented unilaterally in the AAV-5 GFAP GDNF and bilaterally in the AAV-5 SYN GDNF-treated animals. Animals injected intraperitoneally with sodium chloride (NaCl) served as control (A) for the MPTP-treated animals (B, I); i.: virus-injected side n.i.: control side*

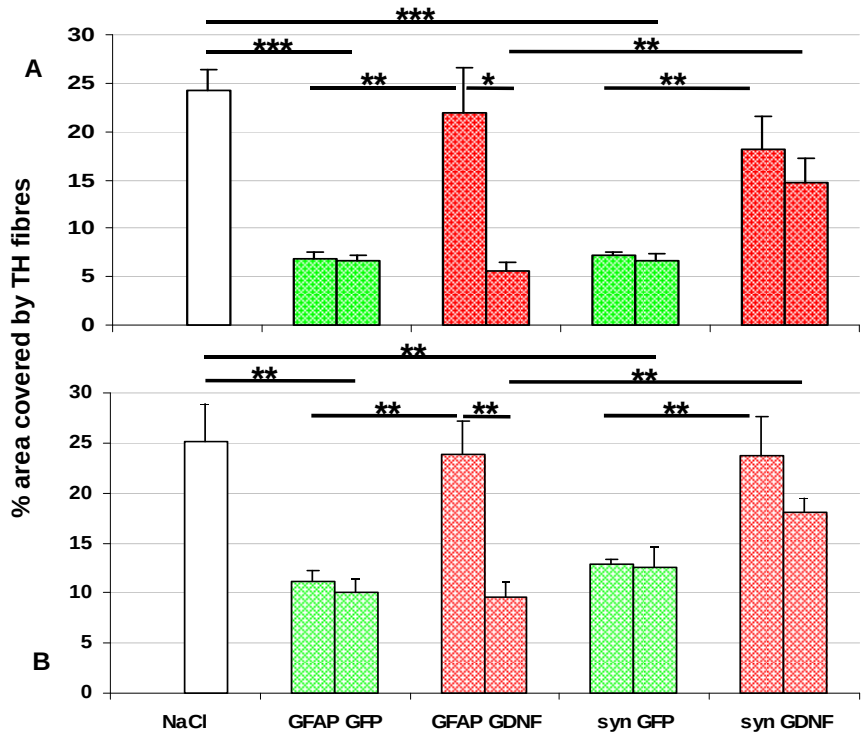

Fig. 5.19: Quantification of TH fibre density in the striatum two weeks (A) and three months (B) after MPTP treatment for 2x10^8 transducing units. *Animals injected intraperitoneally with sodium chloride (NaCl) served as control for the MPTP-treated animals. Bars represent mean ± SD; n = 3-4 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; left bar: virus-injected side; right bar: control side*

Furthermore, a comparison between contralateral control sides two weeks and three months after MPTP treatment reveals a moderate recovery of striatal DA innervation in AAV-5 GFAP GFP, AAV-5 SYN GFP and AAV-5 GFAP GDNF-treated animals (Fig. 5.20). Because at the two weeks time point for neuronal expression of GDNF also the contralateral side was protected, no regeneration was observed three months after MPTP treatment for this condition.

The TH fibre density for the low titre dosage-treated animals has not been determined, because due to restricted animal numbers it was decided to use their *striatum* for the sensitive HPLC measurement of catecholamines (chapter 5.1.3.3).

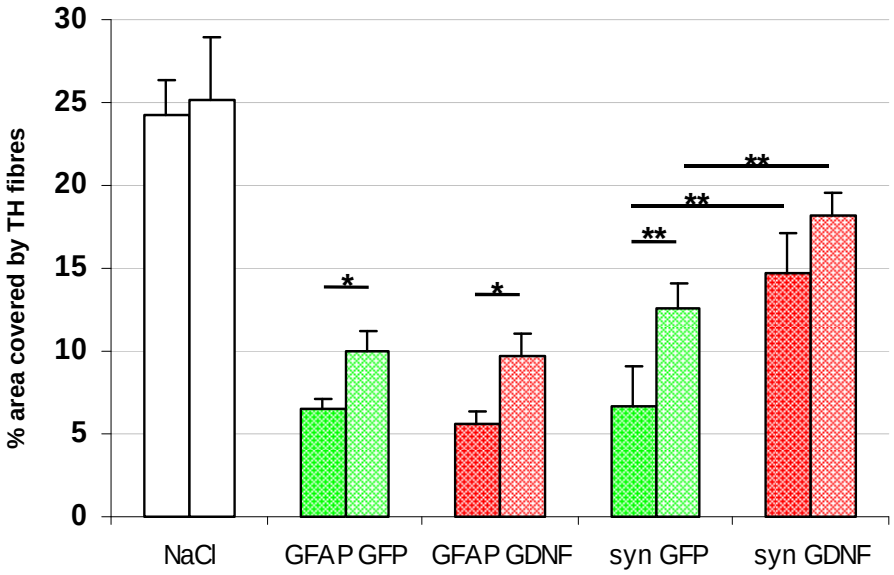

Fig. 5.20: Regeneration of TH fibre density in the striatum for 2x10^8 transducing units. *A comparison between two weeks (first bar) and three months (second bar) fibre density of non-injected control sides shows a significant recovery in AAV-5 GFAP GFP, AAV-5 SYN GFP and AAV-5 GFAP GDNF-treated animals. Bars represent mean ± SD; n = 3-4 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01; left bar: virus-injected side; right bar: control side*

5.1.3.3 Measurement of striatal catecholamine levels

The results revealed by fibre density analysis of the *striatum* for the high titre dosage were supported by the measurement of DA and its metabolites DOPAC and HVA as functional outcome. MPTP induced two weeks after injection a substantial depletion of DA. In addition, the amount of the DA metabolites DOPAC and HVA were decreased (Fig. 5.21 A, C, E). The astrocytic GDNF expression prevented DA, DOPAC and HVA depletion ipsilateral and neuronal GDNF in both hemispheres, although to a significantly higher extent in the ipsilateral *striatum* for HVA (Fig. 5.21 A, C, E).

The degradation of DA to DOPAC and HVA is significantly enhanced in the ipsilateral side of AAV-5 GFAP GDNF-treated animals compared to control levels (DOPAC $p < 0.01$; HVA $p < 0{,}001$). Neuronal GDNF induced only a heightened HVA level in comparison to the control group but for both the ipsi- and contralateral injection side (ipsilateral $p < 0.001$; contralateral $p < 0.01$) (Fig. 5.21 A, C, E).

In contrast to the fibre density analysis three months after MPTP lesion the striatal concentrations of DA, DOPAC and HVA did not recover significantly in comparison to the two week level (Fig. 5.21). The unilateral prevention of astrocytic GDNF against DA, DOPAC and

HVA depletion was still present (Fig. 5.21 B, D, F). Furthermore, neuronal GDNF still protected both hemispheres to the same extent (Fig. 5.21 B, D, F). The primarily increased degradation of DA to DOPAC and HVA was reduced to control level for DOPAC but still significantly enhanced for HVA in the ipsilateral side of AAV-5 GFAP GDNF-treated animals and in both sides for AAV-5 SYN GDNF-treated animals (AAV-5 GFAP GDNF ipsilateral $p < 0.001$; AAV-5 SYN GDNF ipsilateral $p < 0.001$; AAV-5 SYN GDNF contralateral $p < 0.01$) (Fig. 5.21 B, D, F).

The analysis of striatal catecholamine concentrations for the low titre dosage again revealed a depletion of DA and its metabolites two weeks after MPTP treatment (Fig. 5.22 A, C, E). In contrast to the high titre dosage the astrocytic and neuronal GDNF expression prevented DA depletion exclusively ipsilateral (Fig. 5.22 A, C, E). In AAV-5 SYN GDNF-treated animals only DOPAC and HVA levels of the contralateral side were significantly higher compared to the contralateral side of AAV-5 GFAP GDNF-injected animals (Fig. 5.22 A, C, E). In contrast to the situation after high titre treatment exclusively HVA levels are significantly enhanced in the ipsilateral side and only for AAV-5 SYN GDNF-treated animals compared to control levels ($p < 0.01$) (Fig. 5.22 A, C, E).

Three months after MPTP lesion striatal concentrations of DA, DOPAC and HVA for the low titre experiment did not recover in comparison to two week levels (Fig. 5.22). The ipsilateral prevention of astrocytic GDNF against DA, DOPAC and HVA depletion is still present. Neuronal GDNF went back to baseline levels for both hemispheres with a significantly higher extent in the ipsilateral *striatum* only for HVA (Fig. 5.22 B, D, F). Thus, the long-lasting bilateral protective effect of neuronal GDNF, which has been observed for the animals in the high titre experiment, was again established. Since this regeneration of catecholamine levels in the contralateral side of AAV-5 SYN GDNF animals could not be observed in the contralateral side of astrocytic GDNF expression, it could not have been due to normal striatal regenerative capacity, but seems rather to have been dependent on neuronally produced GDNF. The initially increased level of HVA in the AAV-5 SYN GDNF animals was still present three months after MPTP injection in comparison to the control group ($p < 0.01$) (Fig. 5.22 B, D, F).

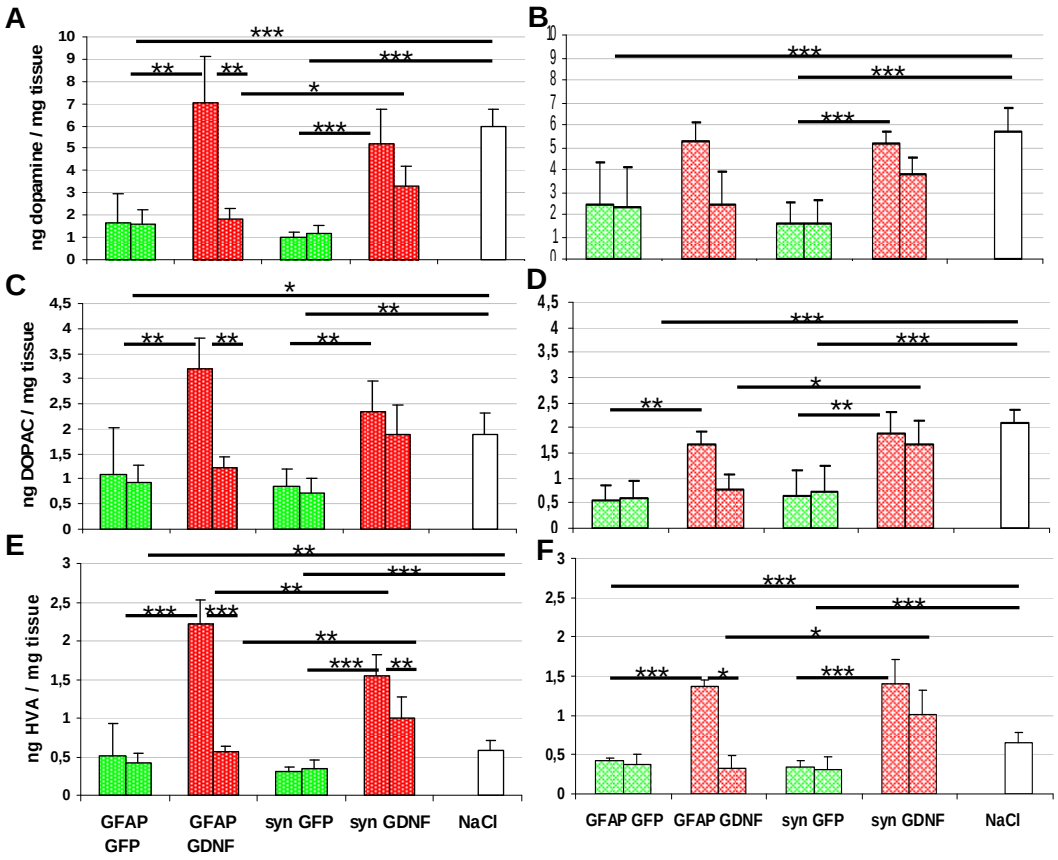

Fig. 5.21: 2x10[8] transducing units: striatal catecholamine concentrations two weeks (A, C, E) and three months (B, D, F) after MPTP treatment. *Bars represent mean ± SD; n = 4-5 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; left bar: virus-injected side; right bar: control side*

Correlating the results of the fibre density analysis and catecholamine concentration measurement in the *striatum* to the ELISA results for GDNF, 812pg GDNF/mg *striatum* seems to be sufficient to deliver a structural and functional protection, whereas 124pg seems not to be a sufficient amount (Fig. 5.6, 5.19, 5.21).

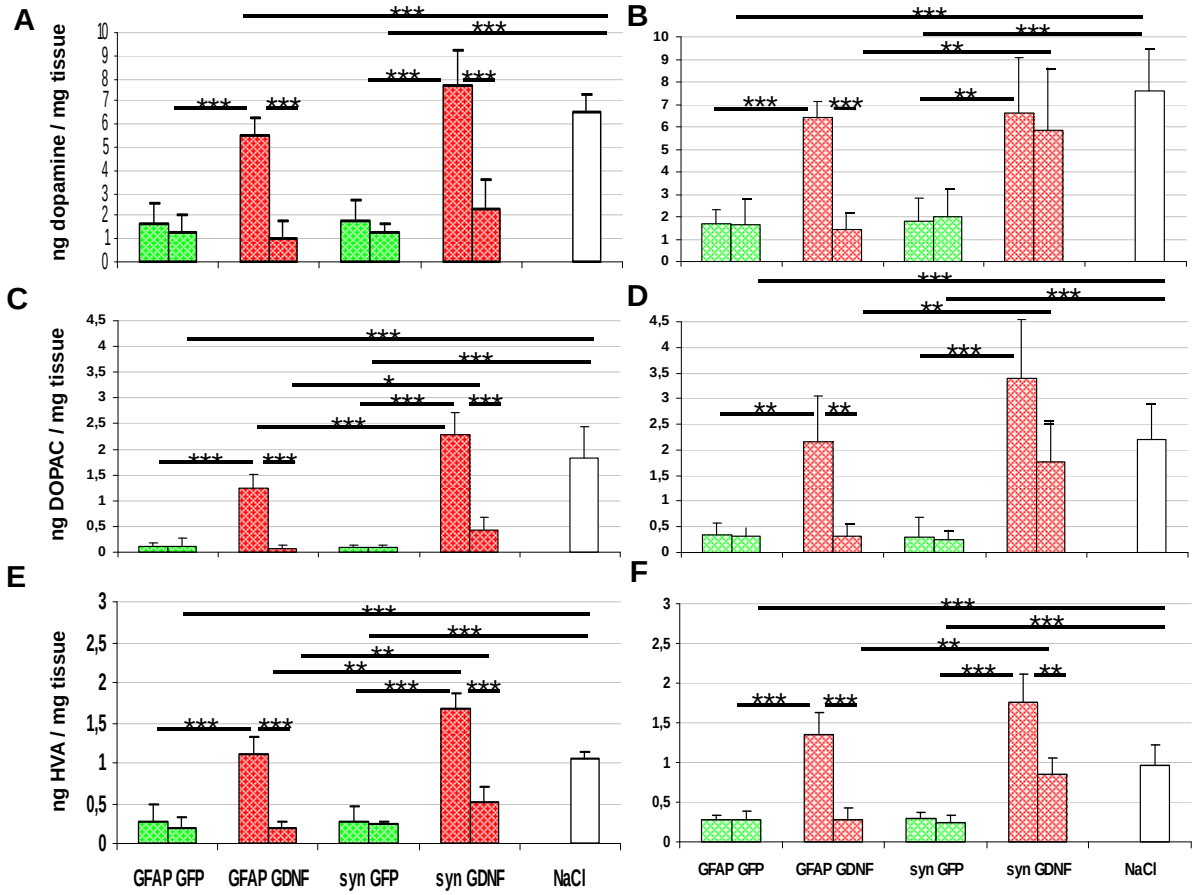

Fig. 5.22: 2x10^7 transducing units: striatal catecholamine concentrations two weeks (A, C, E) and three months (B, D, F) after MPTP treatment. *Bars represent mean ± SD; n = 4-5 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; left bar: virus-injected side; right bar: control side*

5.1.3.4 Functional motor impairments

Because MPTP application in mice leads to degeneration of DA neurons in the SNpc to only about 50% compared with 90% in the 6'OHDA Parkinson model, functional motor impairments are not easy to detect. It was tried to establish differences between experimental groups by performing behavior tests like Open Field, Rotation and Tight rope, without success. No effect of MPTP compared to sodium chloride vehicle i.p. injection was detected as a basis for any further data evaluation (data not shown). The same problem was present during the first Rotarod test with mice under high titre treatment (data not shown). However, finally a significant effect of MPTP for the accelerated rotarod performance of low titre treated mice could be observed (Fig. 5.23). The MPTP-induced impairment of performance in the AAV-5 GFAP GFP and AAV-5 SYN GFP-treated control groups is stable over the period of the experiment for three months. The AAV-5 GFAP GDNF-injected animal groups also worsen in performance directly after MPTP administration but improve over time

and return to baseline level. Animals with neuronal GDNF expression behave in a same way with the exception of being impaired shortly after MPTP treatment merely by trend (Fig. 5.23). A measurement of untreated control animals in parallel to the differently treated groups, showed no alteration in performance (data not shown).

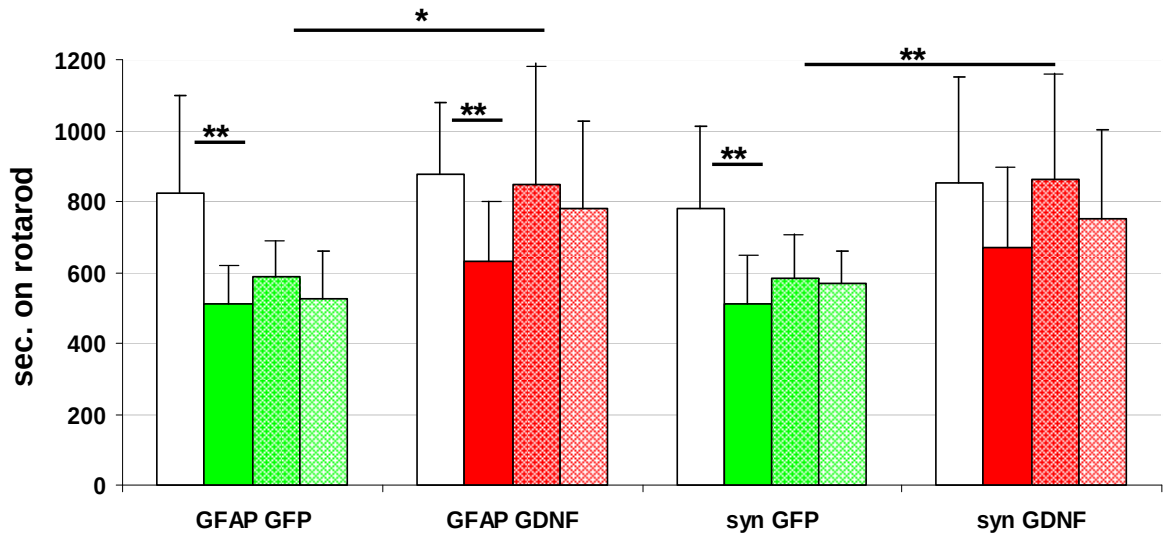

Fig. 5.23: Behavior on rotarod: 2x10^7 transducing units. *Bars represent mean seconds on rotarod ± SD; n = 12 animals for each group (6 animals for the three months time point). Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; first bar: pre MPTP treatment; second bar: one week after MPTP treatment; third bar: two weeks after MPTP treatment; fourth bar: three months after MPTP treatment.*

(i) Long-term neuronal and astrocytic GDNF expression protected ipsilateral DA SNpc neurons to almost 100% from MPTP toxicity, even under conditions of low dosage GDNF.
(ii) Only high amounts of neuronally produced GDNF also protect the contralateral SNpc, arguing for a neuron-specific mechanism activated only by a threshold amount of GDNF.
(iii) Under conditions of high dosage GDNF the MPTP-induced loss of TH-positive fibres in the AAV-5 GFP injected control groups was prevented unilaterally through astrocytic and bilaterally through neuronal GDNF expression.
(iv) Three months after MPTP treatment a moderate recovery of striatal DA innervation was detected.
(v) Long-term neuronal and astrocytic GDNF expression provided ipsilateral protection from DA, HVA, DOPAC depletion in the striatum, even under conditions of low dosage GDNF.
(vi) Both high and low amounts of neuronally produced GDNF also protected the contralateral levels of DA, DOPAC and HVA from depletion in a long-term view.
(vii) Three months after MPTP lesion the striatal concentrations of DA, DOPAC and HVA did not recover significantly, independently of the GDNF concentration.
(viii) Overexpression of GDNF induces an increased metabolism of DA to DOPAC and HVA.
(ix) Regarding rotarod performance, mice were impaired after MPTP treatment and both astrocytic and neuronal produced GDNF protected from that disability with comparable efficiency.

5.2 RET signalling: the basic requirement for GDNF-mediated protection against MPTP toxicity

5.2.1 Astrocytic GFP and GDNF short- and long-term expression in *DAT-Cre, Ret lx* and *DAT Ret $^{lx/lx}$* mice

The striatal injection of 2x10^8 AAV-5 GFAP GFP resulted in a broad spatial GFP expression without differences between genotypes *DAT-Cre, Ret lx and DAT Ret $^{lx/lx}$* for both time points (Fig 5.24 and 5.25). The transduced areas encompass the entire *striatum* and parts of the *cortex*, sparing of the *corpus callosum*. GFP expression was exclusively observed in astrocytes for all three genotypes *DAT-Cre, Ret lx* and *DAT Ret $^{lx/lx}$* (Fig. 5.26).

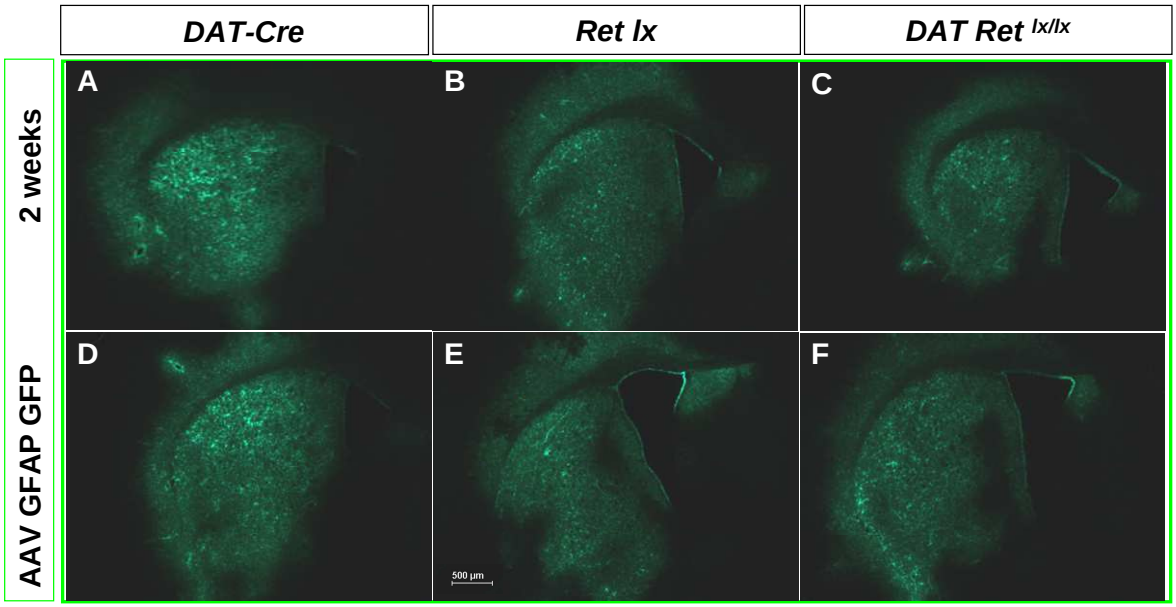

Fig. 5.24: Striatal GFP expression two weeks after AAV-5-mediated gene transfer. *Overview pictures of coronal sections showing anterior (A-C) and posterior (D-F) striatal GFP expression two weeks after AAV-5 infection, expressing GFP under the control of the GFAP promoter for 2x10^8 transducing units in DAT-Cre (A, D), Ret lx (B, E) and DAT Ret $^{lx/lx}$ (C, F) animals. For both the anterior and posterior striatal GFP expression no differences regarding genotypes are observable.*

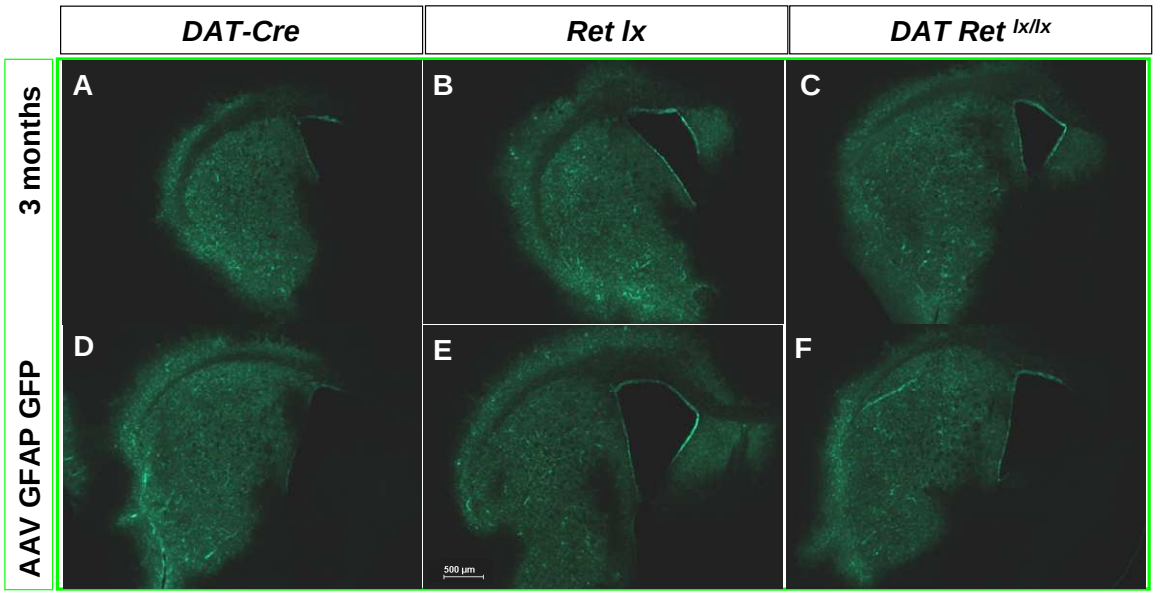

Fig. 5.25: Striatal GFP expression three months after AAV-5-mediated gene transfer. *Overview pictures of coronal sections showing anterior (A-C) and posterior (D-F) striatal GFP expression three months after AAV-5 infection, expressing GFP under the control of the GFAP promoter for $2x10^8$ transducing units in DAT-Cre (A, D), Ret lx (B, E) and DAT Ret $^{lx/lx}$ animals (C, F). For both the anterior and posterior striatal GFP expression no differences in comparison to the two weeks expression level and between genotypes are observable.*

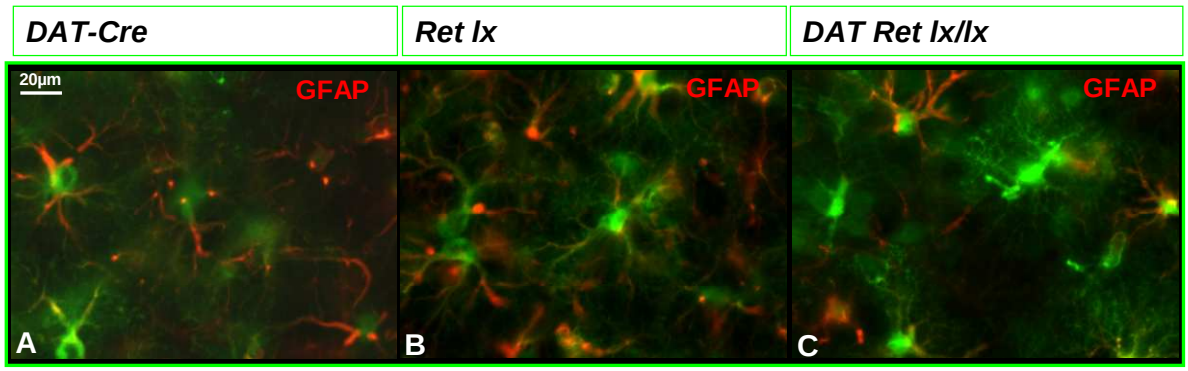

Fig. 5.26: Cell-specific GFP expression after AAV-5-mediated gene transfer (2x10^8 infectious particles, two weeks after infection). *Close-up of the striatum showing AAV-5 GFAP GFP expression exclusively in astrocytes defined by colocalization with GFAP-positive cells (astrocytic marker) for the three genotypes DAT-Cre (A), Ret lx (B) and DAT Ret $^{lx/lx}$ (C).*

The striatal injection of 2x10^8 AAV-5 GFAP GDNF resulted in the same broad spatial GDNF expression without differences between genotypes *DAT-Cre, Ret lx and DAT Ret $^{lx/lx}$* for both time points (Fig. 5.27 M-X). Striatal slices from AAV-5 GFAP GFP-infected mice served as negative control (Fig. 5.27 A-L).

In the SNpc retrograde transport of GDNF from the *striatum* is restricted to the ipsilateral side and remains stable over time without differences between genotypes (Fig. 5.28 M-X). SNpc slices from AAV-5 GFAP GFP-infected mice served as negative control (Fig. 5.28 A-L).

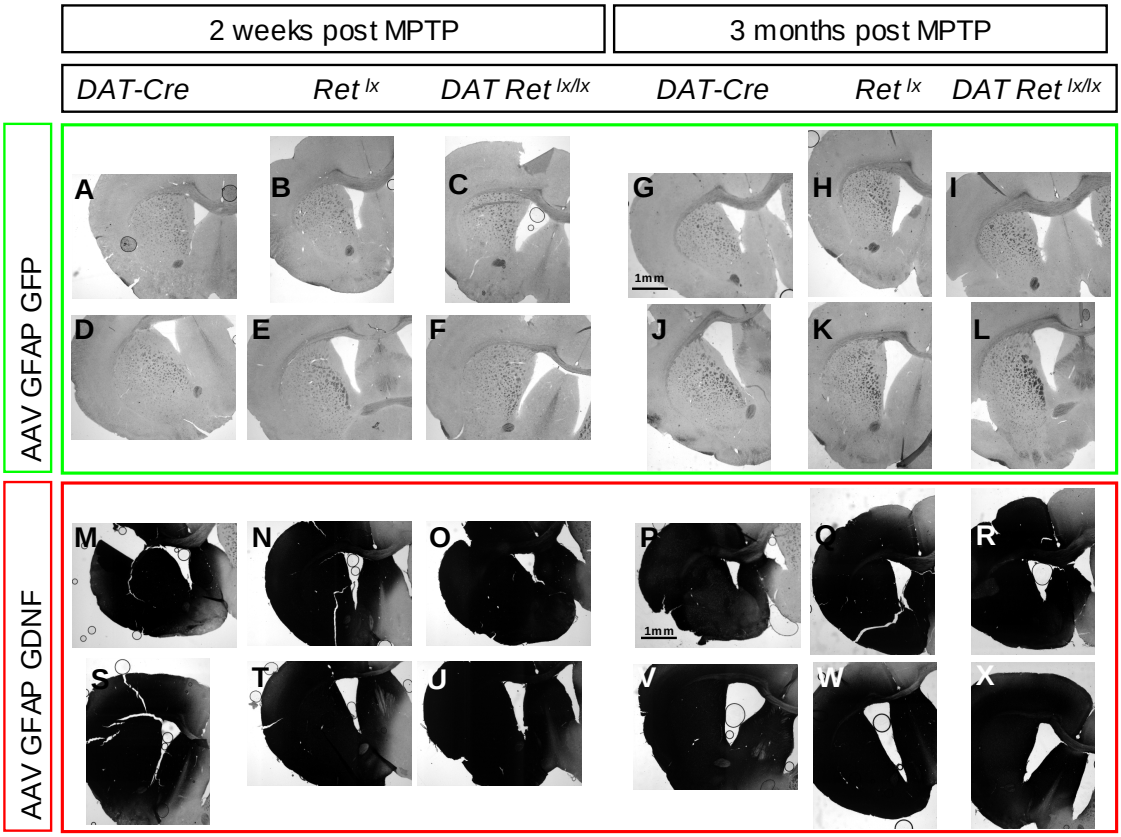

Fig. 5.27: GDNF expression in the striatum of infected mice. *Overview pictures of coronal sections showing GDNF immunoreactivity by DAB staining two weeks (A-F; M-O, S-U) and three months (G-L; P-R, V-X) after unilateral AAV-5 GFAP GFP (A-L) and AAV-5 GFAP GDNF (M-X) infection. Pictures are shown with the injected side of the anterior (A-C, G-I; M-R) and posterior striatum (D-F, J-L; S-U, V-X) on the left for $2x10^8$ transducing units. No differences regarding the negative outcome for GFP-expressing animals and the ipsilateral restricted positive signal for GDNF-expressing animals can be detected between time points and the three genotypes DAT-Cre (A, D, M, S; G, J, P, V), Ret lx (B, E, N, T; H, K, Q, W) and DAT Ret $^{lx/lx}$ (C, F, O, U; I, L, R, X).*

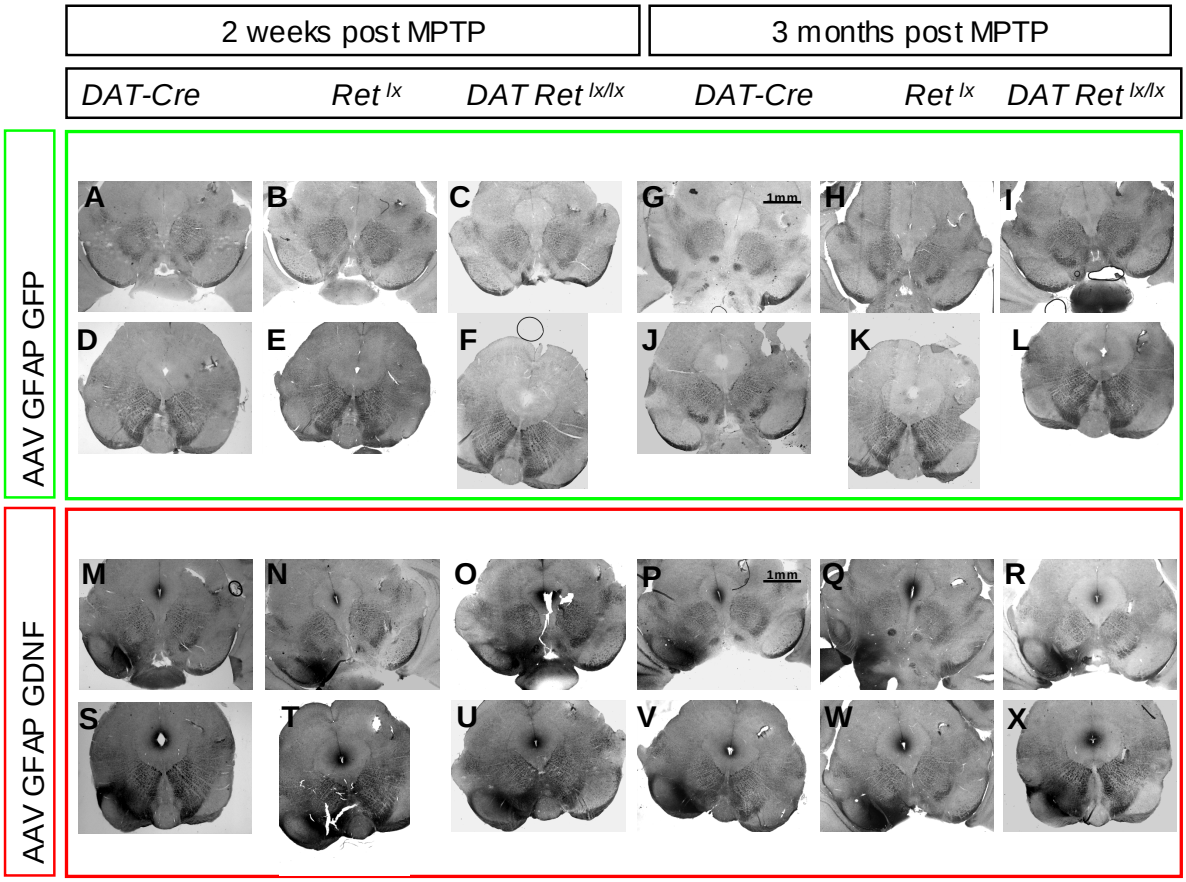

Fig. 5.28: GDNF expression in the SNpc of infected mice. *Overview pictures of coronal sections showing GDNF immunoreactivity by DAB staining two weeks (A-F; M-O, S-U) and three months (G-L; P-R, V-X) after unilateral AAV-5 GFAP GFP (A-L) and AAV-5 GFAP GDNF (M-X) infection. Pictures are shown with the injected side of the anterior (A-C, G-I; M-R) and posterior SNpc (D-F, J-L; S-X) on the left for 2x10^{8} transducing units. No differences regarding the negative outcome for GFP-expressing animals and the ipsilateral restricted positive signal for GDNF-expressing animals can be detected between time points and the three genotypes DAT-Cre (A, D, M, S; G, J, P, V), Ret lx (B, E, N, T; H, K, O, W) and DAT Ret lx/lx (C, F, Q, U; I, L, R, X).*

(i) AAV-5 GFAP GFP and AAV-5 GFAP GDNF injection assured long-term astrocytic transgene expression restricted to the injected side of the striatum and an ipsilateral retrograde transport of GDNF to the SNpc without differences between the genotypes *DAT-Cre, Ret lx and DAT Ret lx/lx*.

5.2.2 GDNF-mediated protection of the nigro-sriatal system against MPTP toxicity in the presence and absence of the RET receptor

AAV-5 GFAP GFP and AAV-5 GFAP GDNF were unilaterally injected into the *striatum* of two-three month old *DAT-Cre*, *Ret lx and DAT Ret* $^{lx/lx}$ mice and two weeks later MPTP was administered subchronically. The unilateral injection of the virus offered the usage of the contralateral side as internal control for each animal. GDNF-mediated neuroprotection against MPTP toxicity was assessed by several means either two weeks or three months after MPTP applications in order to be capable to differentiate between acute and regenerative effects of GDNF: stereological quantification of DA SNpc neurons, quantitative determination of TH positive striatal fibre density (cooperation partner Dr. Edgar Kramer, Center for Molecular Neurobiology Hamburg (ZMNH)) and identification of striatal levels of DA and its metabolites DOPAC and HVA.

5.2.2.1 Quantification of DA SNpc neurons by stereology

Stereological quantification of nigral DA neurons revealed that expression of GFP in astrocytes had no effect compared with the contralateral side for both time points and all genotypes (Fig. 5.29 D-F, Fig. 5.30 D-F and Fig. 5.31). Two weeks after MPTP administration astrocytic expression of GDNF in *DAT-Cre* and *Ret lx* mice resulted in a significant ipsilateral neuroprotection, but there was no protection in the contralateral SNpc (Fig. 5.29 G-H and Fig. 5.31 A). In contrast, GDNF did not show any protective effect to the SNpc DA neurons of *DAT Ret* $^{lx/lx}$ mice, in which the RET receptor is missing (Fig. 5.29 I and Fig. 5.31 A).

At three months after MPTP administration again only the SNpc of *DAT-Cre* and *Ret lx* control mice was ipsilateral protected by astrocytic GDNF expression (Fig. 5.30 G-H and Fig. 5.31 B). DAT Ret $^{lx/lx}$ mice were completely hit by MPTP toxicity with no difference between them and the AAV-5 GFAP GFP- treated animals (Fig. 5.30 I and Fig. 5.31 B).

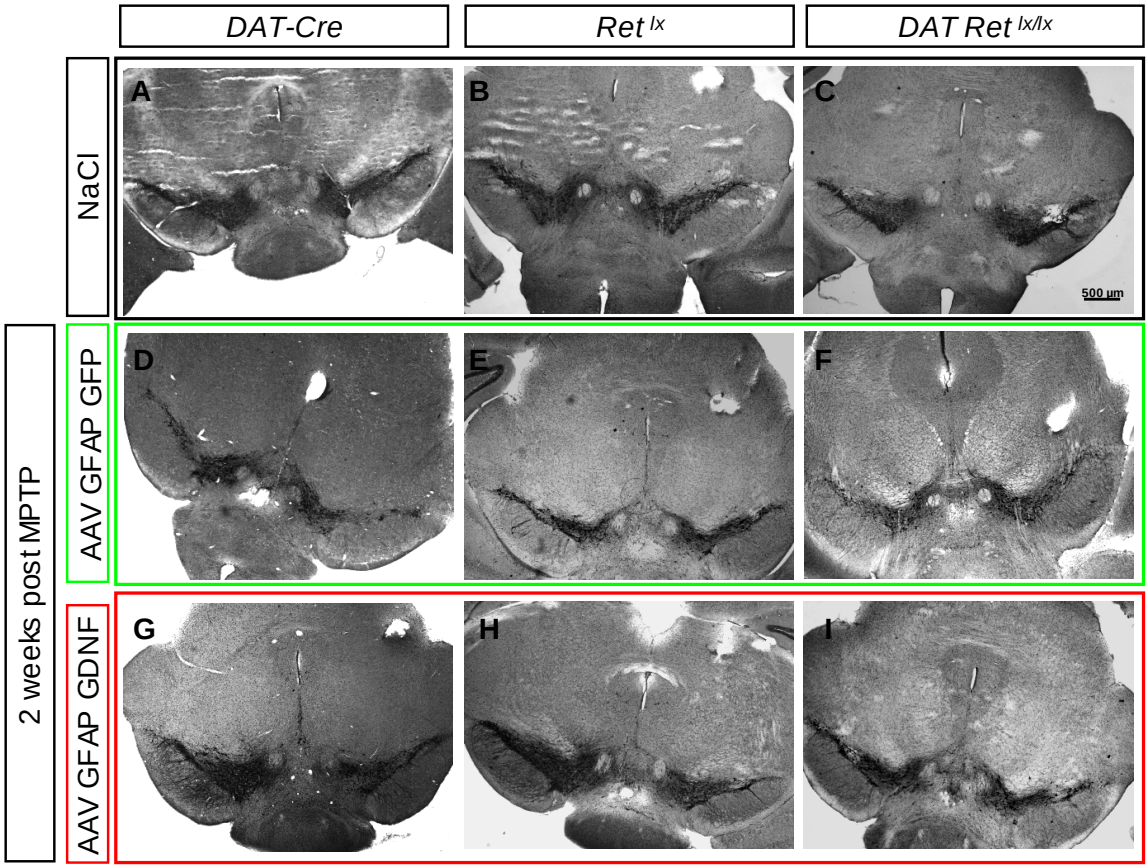

Fig. 5.29: DA SNpc neurons two weeks after MPTP treatment. *Overview of coronal sections stained for DA neurons of the SNpc by TH/DAB and Nissl. The MPTP-induced loss of SNpc neurons in all three AAV-5 GFAP GFP-injected control groups (D-F) can be prevented ipsilaterally in the AAV-5 GFAP GDNF (G-I) -treated DAT-Cre (G) and Ret lx (H) animals but not for the DAT Ret $^{lx/lx}$ (I) group. Animals injected intraperitoneally with sodium chloride (NaCl) served as control (A-C) for the MPTP-treated animals (D, I). left: virus-injected side for 2x10^{8} transducing units; right: control side*

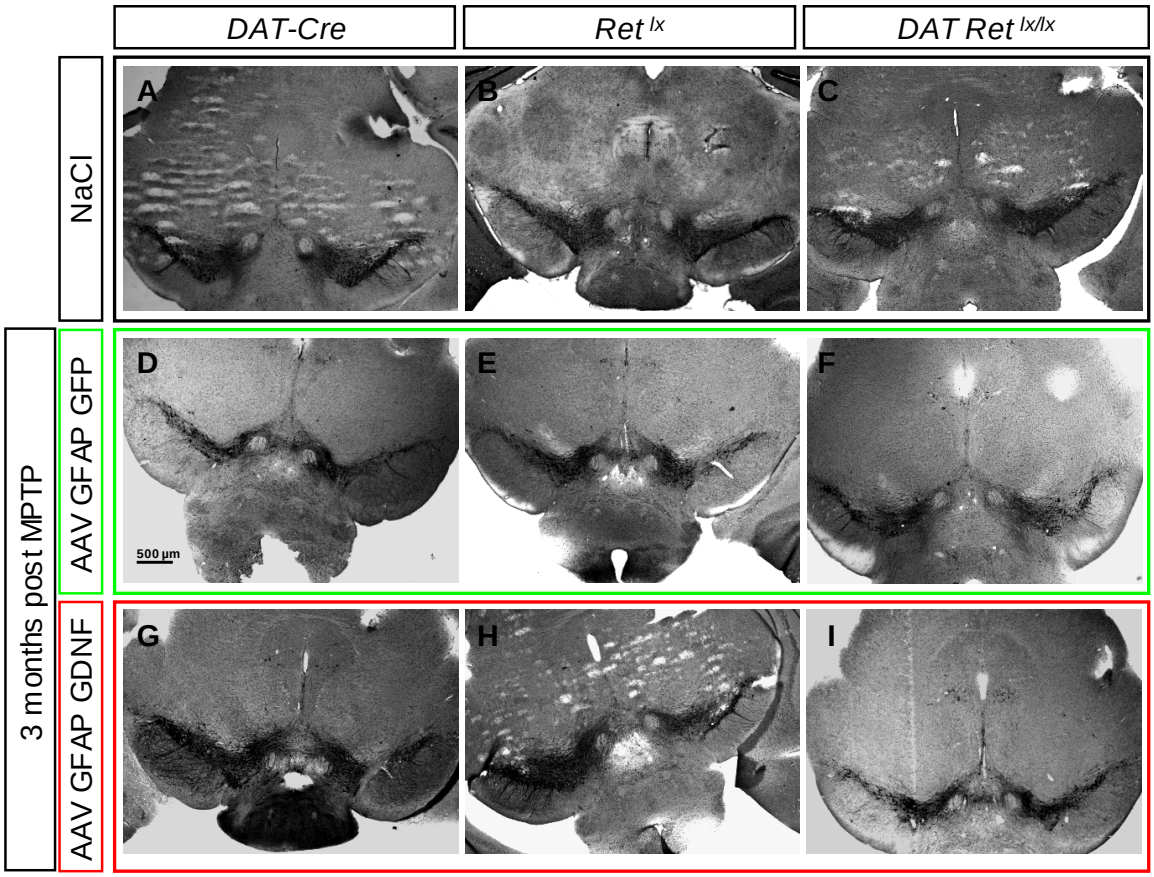

Fig. 5.30: DA SNpc neurons three months after MPTP treatment. *Overview of coronal sections stained for DA neurons of the SNpc by TH/DAB and Nissl. The MPTP-induced stable loss of SNpc neurons in all three AAV-5 GFAP GFP-injected control groups (D-F) can be prevented long-term ipsilaterally in the AAV-5 GFAP GDNF (G-I) -treated DAT-Cre (G) and Ret lx (H) animals but not in the DAT Ret $^{lx/lx}$ (I) group. Animals injected intraperitoneally with sodium chloride (NaCl) served as control (A-C) for the MPTP-treated animals (D, I). left: virus-injected side for 2x10^{8} transducing units; right: control side*

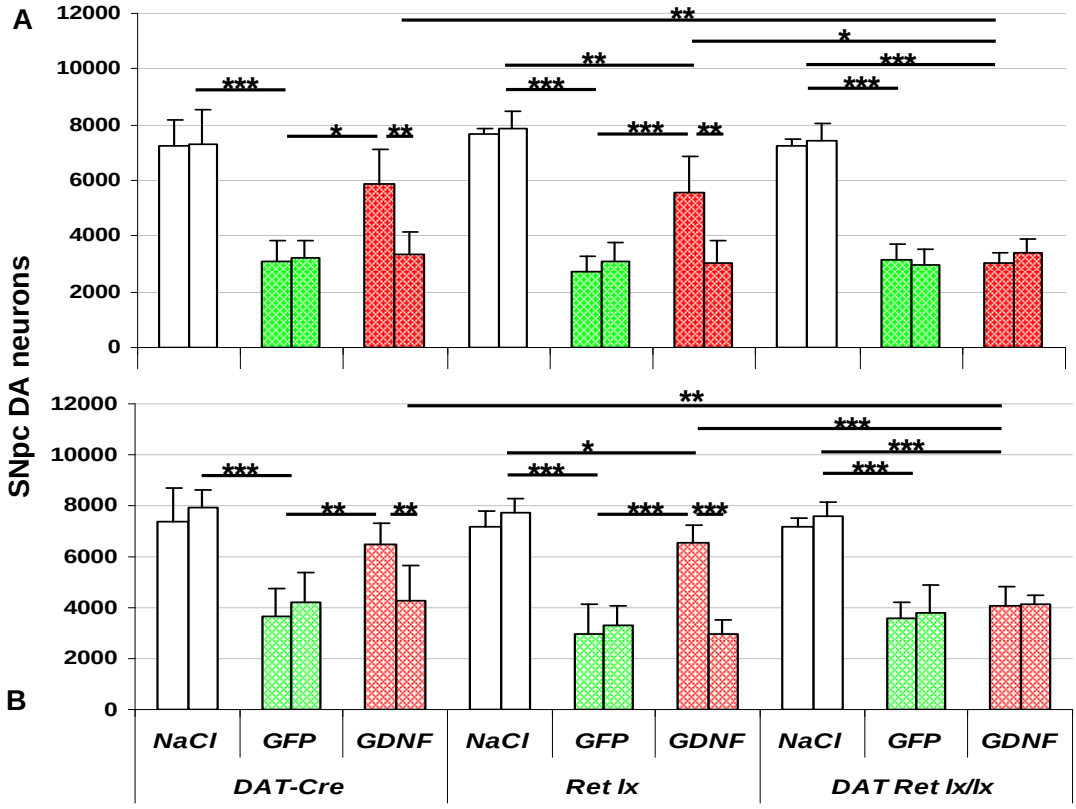

Fig. 5.31: Stereological cell counts for TH and Nissl-positive cells in the SNpc two weeks (A) and three months (B) after MPTP treatment. *Animals injected intraperitoneally with sodium chloride (NaCl) served as control for the MPTP-treated animals. Bars represent mean ± SD; n = 3-6 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; GFP: AAV-5 GFAP GFP-injected animals (2x10^8 transducing units); GDNF: AAV-5 GFAP GDNF-injected animals (2x10^8 transducing units); left bar: virus-injected side; right bar: control side*

5.2.2.2 Quantification of striatal catecholamine concentrations by HPLC

Two weeks after MPTP treatment HPLC analysis revealed a substantial depletion of DA and its metabolites DOPAC and HVA without differences between genotypes (Fig. 5.32). Astrocytic GDNF expression prevented DA, DOPAC and HVA depletion ipsilateral in *DAT-Cre* and *Ret lx control* mice but not in *DAT Ret $^{lx/lx}$* mice (Fig. 5.32). The degradation of DA to DOPAC and HVA was significantly enhanced in the ipsilateral side of AAV-5 GFAP GDNF-treated *DAT-Cre* and *Ret lx* animals compared to control levels. This increased DA metabolism rate was not detected in the *DAT Ret $^{lx/lx}$* animals (Fig. 5.32 B, C).

Three months after MPTP lesion the striatal concentration of DA, DOPAC, and HVA recovered in comparison to the two week level in *DAT-Cre* and *Ret lx* animals (Fig. 5.33 and 5.34). The ipsilateral protection of DOPAC and HVA levels in both control strains as well as the increased metabolism rate could still be observed. Reproducing already published data,

striatal catecholamine levels of *DAT Ret* $^{lx/lx}$ animals did not recover at all (Fig. 5.33 and 5.34) (Kowsky et al., 2007).

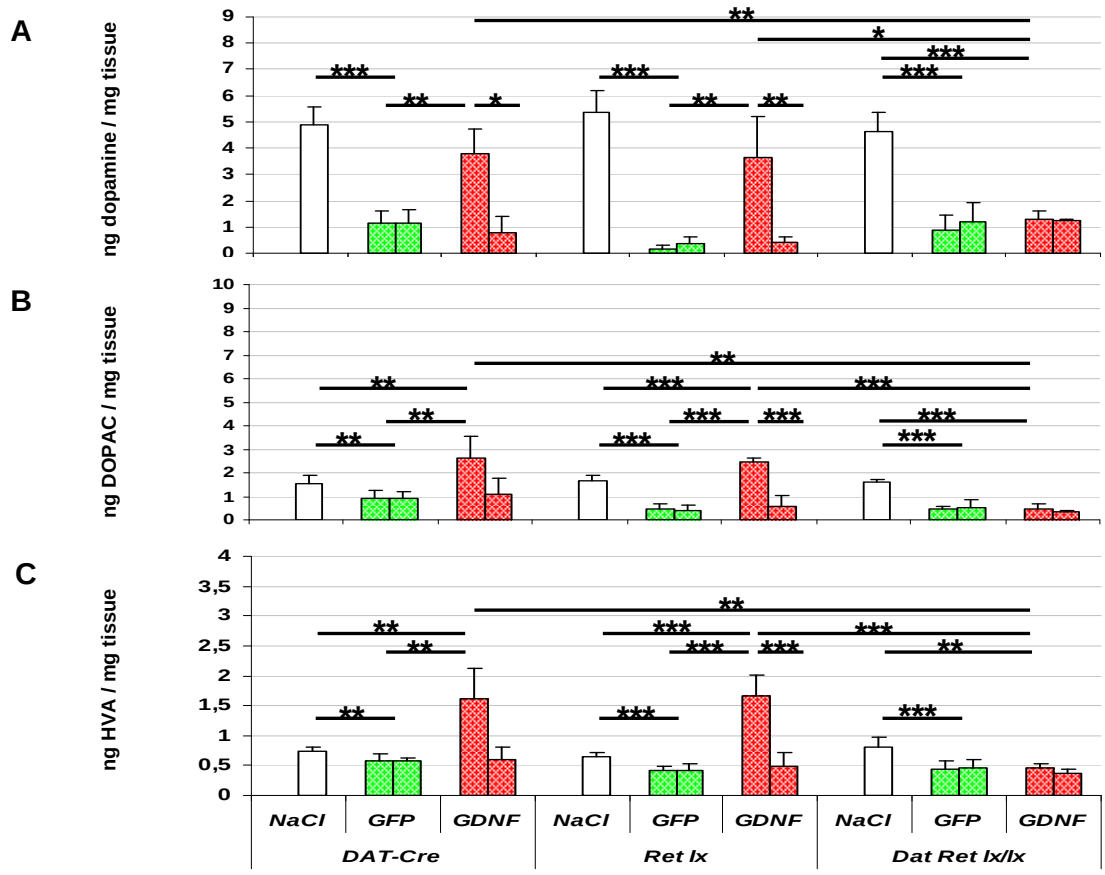

Fig. 5.32: Striatal catecholamine concentrations two weeks after MPTP treatment. *Bars represent mean ± SD; n = 3-6 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; GFP: AAV-5 GFAP GFP-injected animals (2x10*^{8} *transducing units); GDNF: AAV-5 GFAP GDNF-injected animals (2x10*^{8} *transducing units); left bar: virus-injected side; right bar: control side*

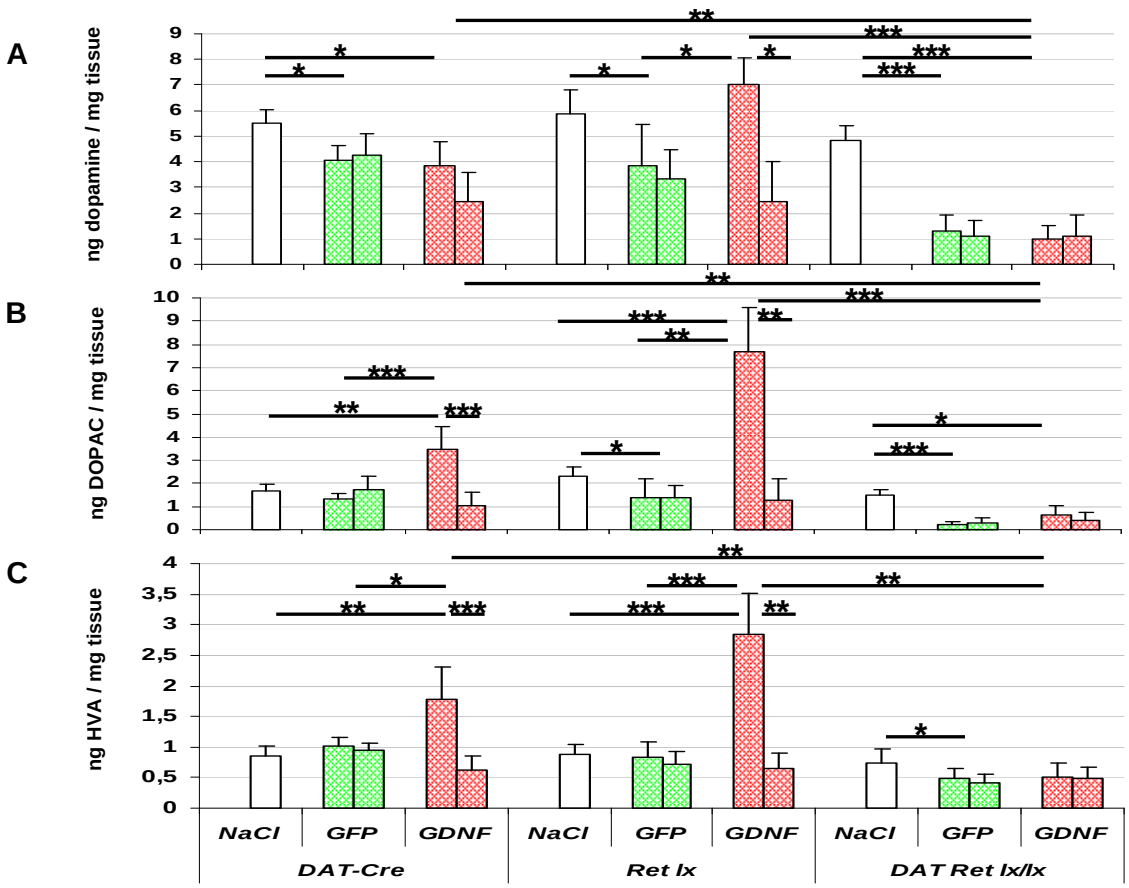

Fig. 5.33: Striatal catecholamine concentrations three months after MPTP treatment. *Bars represent mean ± SD; n = 3-6 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01, p***< 0.001; GFP: AAV-5 GFAP GFP-injected animals (2x10^8 transducing units); GDNF: AAV-5 GFAP GDNF-injected animals (2x10^8 transducing units); left bar: virus-injected side; right bar: control side*

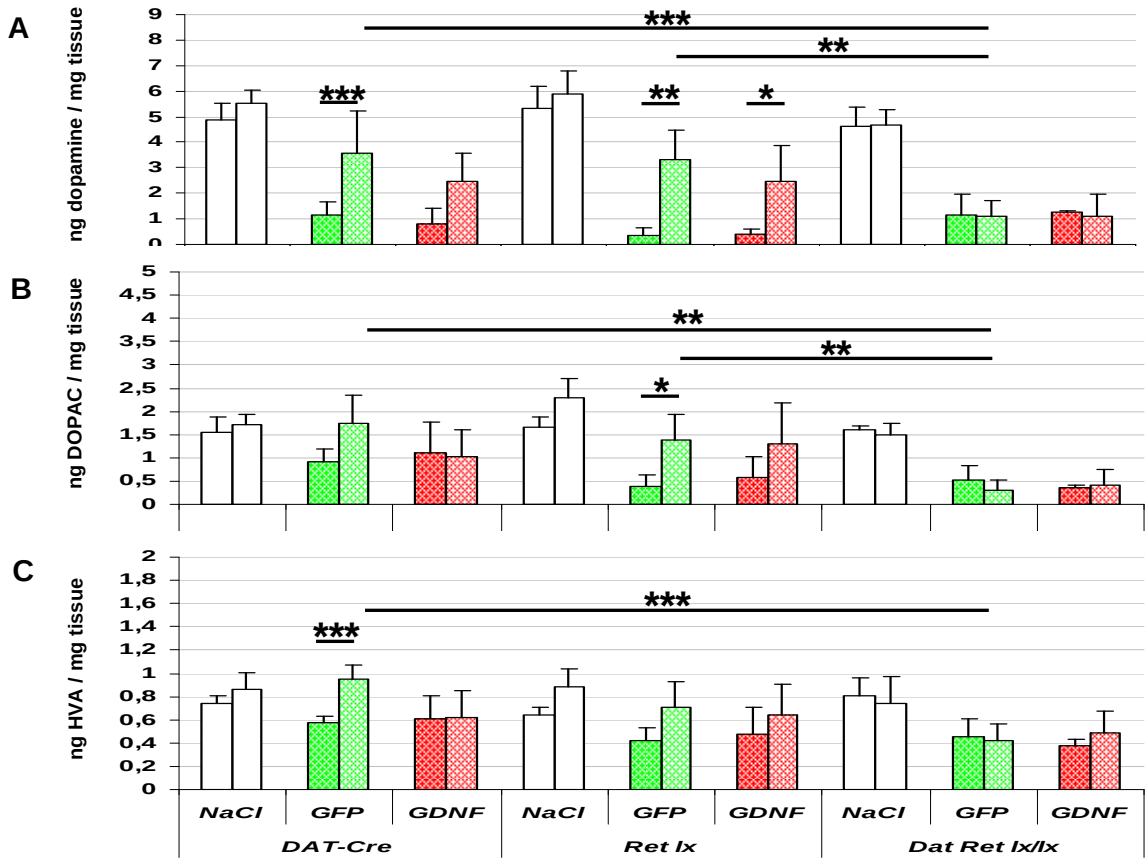

Fig. 5.34: Regeneration of striatal catecholamine concentrations. *A comparison between two weeks (first bar) and three months (second bar) striatal catecholamine concentrations of non-injected control sides is shown. Bars represent mean ± SD; n = 3-6 animals for each group. Comparisons were made by ANOVA followed by Tukey's post hoc test. p* < 0.05, p** < 0.01; GFP: AAV-5 GFAP GFP-injected animals (2x10^8 transducing units); GDNF: AAV-5 GFAP GDNF-injected animals (2x10^8 transducing units); left bar: virus-injected side; right bar: control side*

(i) Astrocytic GDNF expression mediates short- and long-term protection of DA SNpc neurons from MPTP toxicity if they express the RET receptor.

(ii) Astrocytic GDNF expression prevented DA, DOPAC and HVA depletion after MPTP intoxication ipsilateral in *DAT-Cre* and *Ret lx control* mice but not in *DAT Ret* $^{lx/lx}$ mice.

(iii) Three months after MPTP lesion the striatal concentration of DA, DOPAC, and HVA recovered in *DAT-Cre* and *Ret lx* animals but not in *DAT Ret* $^{lx/lx}$ mice.

There is no alternative to an intact GDNF-RET mediated signalling in the nigrostriatal sytem, this being a prerequisite for the protective potential of GDNF and the natural regenerative capacity of the striatum after MPTP lesion in mice.

6. Discussion

6.1 Astrocytes or neurons as source for GDNF-mediated protection of the nigrostriatal system against MPTP toxicity mice

6.1.1 Transduction properties of the AAV-5 serotype

Wild-type (wt) AAV is not known to be associated with any disease in humans or mammals and shows no inherent vector toxicity. A short viral production time and long-lasting transgene expression from the recombinant genome are further benefits of these vector systems (Shevtsova et al., 2005; Kügler et al., 2003; Peel and Klein, 2000). Thus, recombinant AAV vectors are considered to have one of the highest biosafety rankings among viral vectors (Mandel et al., 2008). The most extensively studied serotypes are AAV-2, the only AAV serotype in active clinical trials, and AAV-5 (Hildinger and Auricchio 2004). This strategy has been challenged recently by the failure of AAV-2 based vectors to escape human immune responses in patients with pre-existing immunity to AAV-2 (Hasbrouck and High et al., 2008). It is currently unknown if such pre-existing immunity, which is present in the majority of humans, might impact on trials delivering the vector into the immuno-specialized brain. It is also unknown if failure of the GDNF-relative neurturin expressed from an AAV-2 vector in the brain of PD patients was due to immune related problems or to an inefficiency of the protein (Bartus 2009, Scientific Symposium 113: Late stage industry clinical trials). In any case, of all known AAV serotypes the serotype-5 capsid is the least homologous to AAV-2 and pre-clinical studies have already demonstrated that AAV-5 can be re-delivered into rodent CNS in animals pre-immunized against AAV-2 (Peden et al., 2004). It appears that cells transduced via AAV-2 present capsid peptides for a much longer period than other serotypes (Lowenstein et al., 2007; Thomas et al., 2009). Indeed, there is apparently little to no brain immune response to the AAV-5 capsid (Manfredsson et al., 2009). Thus, AAV-5 based vectors may hold great promise for clinical applicability. In this study the AAV-5 capsid provoked an astroglial reaction (detected via GFAP staining), which could be reduced to a weak, short-lasting effect by decreasing the viral titre (Fig. 5.7-5.10). GFAP is the principal intermediate filament in mature astrocytes of the CNS and a member of the cytoskeletal protein family. It is thought to be important in modulating astrocyte motility and shape by providing structural stability to astrocytic processes. In the CNS of higher vertebrates, following an injury astrocytes become reactive and respond with astrogliosis, characterized by a rapid synthesis of GFAP (Eng et al., 2000). The observed recovery from astrogliosis three months after stereotaxic injection argues against chronic activation of astrocytes (Fig. 5.9-5.10). Since simply the stereotaxic injection of sodium chloride caused astrogliosis, only slightly enhanced in the presence of a non-expressing AAV-5, the effect of AAV-5 itself on astrogliosis has to be ranked as cumulative but non-initiative.

Activation of microglia due to inflammation can be detected by staining against Iba-1, which is specifically expressed in microglia. The detection of Iba-1 upregulation makes it possible to discriminate between resting and activated microglia. Upregulation of Iba-1 could be selectively but weakly and only detected in the acute phase after GFP and GDNF expression in astrocytes. No such reaction was detectable after neuronal transgene expression or after transduction with a vector not expressing a transgene. Since both EGFP and GDNF expression provoked micgroglial activation, no potentially beneficial or detrimental effect of this activation could have influenced the outcome of this study (Fig. 5.11-5.12).

One of the major causes of the inconsistend outcome of GDNF in clinical trials has been suggested to relate to the distribution of GDNF in the putamen by different infusion techniques (Manfredsson et al., 2009). Some of the investigators involved in these clinical studies have recently stated that gene transfer is likely a potentially better alternative delivery method for GDNF (Peterson and Nutt 2008). Since AAV-5 allows for a drastically increased spread of the vector compared to AAV-2 (Shevtsova et al., 2005), the application of AAV-5 might circumvent the problem. Indeed, in this study stereotaxic injection of AAV-5 resulted in long-lasting, broad spatial distribution of transduced cells over the entire *striatum*, even after a 10-fold reduction of the viral titre, but with reduced transgene expression per cell and less density of transduced cells (Fig. 5.1 and 5.2).

6.1.2 Cell type-specific expression through the control of the GFAP or synapsin promoter

The possibility of implementing a transgene expression exclusively in target cells based on cell specific promoters is a further benefit of AAV vector systems (Shevtsova et al., 2005). The type of cells transduced can play an important role for the GDNF effect and astrocytes, which perform many functions critical for neuronal survival, are very promising candidates for GDNF delivery (Sandhu et al., 2009; Jakobsson et al., 2004; Byrd et al., 2004; Dringen and Hirrlinger 2003; Wilson et al., 1997).

The various AAV serotypes differ in structure and bind unique membrane-associated receptors, resulting in differences in cell tropism. The AAV-5 vector was found to be able to transduce both neurons and astrocytes, being only transcriptionally targeted to either one of these cell types in a very efficient way (Shevtsova et al., 2005). The results of this study support these data by demonstrating that AAV-5 tropism is not restricted to neurons as in the case for the AAV-2 serotype. In contrast, astroglial cells are transduced with a very high efficacy given that transgene expression was driven by the GFAP promoter (Fig. 5.3 A). Efficient transduction of astrocytes by AAV vectors has been demonstrated only very recently but is still hampered by considerable co-expression in neurons (Koerber et al., 2009) and the need for either very high vector dosage (Lawlor et al., 2009) or systemic vector application in

neonates (Foust et al., 2009). In contrast, the AAV-5 based strategy presented here demonstrated very low vector dosages to be still highly efficient in astrocytic transgene expression, while high titres were still entirely astrocyte-specific. Neurons are transduced specifically and with a very high efficacy given that transgene expression was driven by the SYN promoter (Fig 5.3 B), without restrictions for low vector dosages.

6.1.3 Cell type-specific expression of GDNF: impacts on GDNF transport and distribution within the brain

It was hypothesised that GDNF produced by astrocytes might be as effective as neuronal GDNF. Although neurons are the physiological cellular basis of striatal GDNF expression during development of the brain (Oo et al., 2005), astrocytes become the predominant source of GDNF in the adult brain upon injury (Bresjanac and Antauer 2000; Nakagawa and Schwartz 2004; Chen et al., 2006).

GDNF is transported retrogradely from the *striatum* to the DA cell bodies in the SNpc (Barroso-Chinea et al., 2005; Oo et al., 2005; Kordower et al., 2000; Choi-Lundberg and Bohn 1995; Tomac et al., 1995). A quantitative GDNF determination by ELISA two weeks upon viral injection in the *striatum* revealed that the transport of GDNF to the ipsilateral SN was much more efficient if GDNF was produced by neurons for both high and low viral titres (Fig 5.6). In the ipsilateral *striatum* only little differences in the amount of GDNF were detected after high and no differences upon injection of low viral titres (Fig. 5.6).

AAVs can also be transported retrogradely (Kaspar et al., 2003; Pirozzi et al., 2006). However, a comparison of the retrograde transport efficiency between serotypes revealed AAV-1 to have the greatest efficiency after intramuscular injection. Serotypes 2-6 showed a low efficiency for retrograde transport (Hollis et al., 2008). Thus, in this study the presence of GDNF in the SN after striatal virus injection is probably not due to the retrograde transport of the AAV-5.

In the case of high GDNF expression levels contralateral nigrostriatal structures showed increased GDNF levels; GDNF produced by neurons showed a tendency to a more efficient transport to the contralateral *Striatum* than GDNF produced by astrocytes, probably due to the fact that neurons are able to secrete GDNF not only from their cell bodies but also from pre-synaptic sites, which can be found at remote areas, i.e. the contralateral hemisphere.

Thus, in addition to transport of GDNF from the ipsilateral *striatum* to the ipsilateral SNpc, tracks others than those, namely from the ipsilateral to the contralateral *striatum* and SN or from the SN to the *striatum* might be in use in the presence of high amounts of GDNF. Indeed, anterograde transport of GDNF to the *striatum* after nigral AAV injection (Arvidsson et al., 2003; Kordower et al., 2000) and contralateral effects of high amounts of GDNF were described previously (Peterson and Nutt 2008; Slevin et al., 2007; Eslamboli et al., 2005).

The explanation of how GDNF reaches all the remote sites in this experimental setup is currently a suggestion only. However, GDNF expressed by neurons was detected to a strong extent in areas outside the SNpc, e.g. the SNpr (Fig. 5.4 H/J and Fig. 5.5 F/H), an indication for an anterograde delivery of GDNF to the midbrain.

6.1.4 Astrocytic versus neuronal GDNF: Which cellular source of GDNF is favourable for a PD gene therapy?

The results of this study suggest that expressing the neurotrophin GDNF by neurons is more favourable than expression of GDNF by astrocytes in an established animal model of PD. Even under low dosage GDNF a long-term prevention of the MPTP-induced reduction in DA neurons of the SNpc, DA positive fibres and catecholamine levels in the *striatum* was effected unilaterally through astrocytic and bilaterally through neuronal GDNF expression (Fig. 5.14, 5.16, 5.19, 5.21, 5.22). Possible actions by which GDNF could provide positive effects on the nigrostriatal system in the model of this study are 1) promotion of survival for remaining neurons; 2) restoration of neuronal function regarding the DA phenotype; 3) induction of DA neurogenesis in the SN; or 4) stimulation of the DA system (Peterson and Nutt 2008). However, the first hypothesis is most likely the protective effect of GDNF (reviewed by Manfredsson et al., 2009).

Although delivery of bilateral nigrostriatal protection by neuronal expression of GDNF appears to be an approach with high therapeutic efficacy, it reveals serious safety concerns.

Under normal conditions GDNF shows highest expression levels during development and greatly reduced expression in the adult brain (Saavedra et al., 2008). Adult catecholaminergic neurons crucially depend on GDNF signalling in the adult brain (Pascual et al., 2008) and the prototype receptors GFRα and RET are expressed throughout the adult brain (Sarabi et al., 2003; Glazner et al., 1998). Furthermore, GDNF has been proposed to be able to induce signalling through additional receptor systems like NCAM and integrins (Sariola and Saarna 2003).

The hypothesis that GDNF probably only induces beneficial effects on remaining nigrostriatal DA neurons, led to the conclusion regarding the failure of clinical trials that the patients in those trials likely had too little of their nigrostriatal dopaminergic tract intact for the GDNF to induce therapeutic effects. Performance of future Phase II trials in earlier stage patients or higher and effectively distributed GDNF amounts would be a better test of the clinical efficacy for GDNF in PD (Manfredsson et al., 2009; Peterson and Nutt 2008). However, currently gene therapy is an irreversible process and should the putative need for higher dosages of GDNF and the wish to target younger patients in order to develop a successful strategy for Parkinson's patients be realized, serious side effects may be imposed by the dissemination of a potent neurotrophic factor to areas outside the affected nigrostriatal system

(Manfredsson et al., 2009). The detailed wiring of the human brain has not at all been fully elucidated (Guye et al., 2008)**,** and it is unfeasible to predict into which distant CNS nuclei caudate and putamenal neurons may project and deliver GDNF after being transduced by a neuronal gene therapy vector. Further, the long-term presence of a neurotrophic factor may result in an activation of low affinity receptors, in turn inducing unforeseeable signalling events. For example, in monkey, cat and mouse the nigro-collicular projection originates in the substantia nigra pars reticulata, links the *basal ganglia* to the sensorimotor layers of the superior colliculus and is crucially involved in promotion of orienting behaviour (Kaneda et al., 2008; Harting et al., 2001; Huerta et al., 1991). The functional capabilities of these neurons might eventually be modified if exposed long-term to a potent neurotrophic factor as seen after neuronal GDNF expression in the *striatum*. Also, weight loss, a common side effect in a number of clinical trials and currently the best-characterized off-target effect of GDNF, has recently been suggested as being caused by the secretion of neuron-expressed GDNF from un-myelinated axons in the medial forebrain bundle and activation of hypothalamic corticotriopin releasing hormone secreting neurons (Manfredsson et al., 2009).

In contrast, in no case did striatal astrocyte-derived GDNF exert measurable biological effects outside the target structures (ipsilateral *striatum* and nigra), not even in the case of injection of high titre of virus. Astrocyte-derived GDNF could only be transported retrogradely, resulting in only 10% of the total striato-nigral GDNF transport as compared to neuronal expression. None-the-less, astrocyte-derived GDNF was as efficient as neuron-derived GDNF in preventing degeneration of nigral DA neurons, their striatal terminals and their striatal DA content from MPTP toxicity. Thus, in the ipsilateral nigrostriatal projection the astrocytic expression of GDNF provided an essential safety criterion by avoiding remote biological effects without any restriction on therapeutic efficacy. While lowering the vector titre somewhat improved the safety profile of neuronal GDNF expression, it still resulted in a pronounced impact on contralateral neurotransmitter synthesis. At two weeks after MPTP a four-fold increase of GDNF over control levels in the contralateral nigra and a two-fold increase in the contralateral *striatum* showed no benefit but were sufficient to fully restore striatal DA levels after long-term (three months) expression, demonstrating that some biological effects of GDNF may take their time to build up. In contrast, after astrocytic GDNF expression, a 13-fold increase of GDNF in the ipsilateral nigra was fully protective (low titre group), but the same increase in GDNF levels did not show any biological effect in the contralateral nigra (in the high titre group), exemplifying that it is not only the absolute level of GDNF which might exert biological effects but also the mode of delivery (Fig. 5.6, 5.14 and 5.16). Interestingly, retrogradely transported GDNF is widely accepted to have protective effects (reviewed by Kirik et al., 2004), while anterogradely transported GDNF does not have any protective potenial or is even neurotoxic (Arvidsson et al., 2003; Kordower et al., 2000).

This fact leads to the assumption of a modifying mechanism, depending on the mode of GDNF transport, which influences the protective capacity of GDNF.

Three months after MPTP treatment a moderate recovery of striatal DA innervation was detected (Fig. 5.20), whereas no such recovery was observed regarding striatal concentrations of DA, DOPAC and HVA for all MPTP treated animal groups (Fig. 5.21, Fig. 5.22). It is known that structural striatal recovery starts earlier than functional recovery of catecholamine levels (Mitsumoto et al., 1998). Thus, in this model striatal recovery was indeed already initiated but obviously had not enough time to show its full regenerative capacity.

Overexpression of GDNF induced increased levels of DOPAC and HVA (Fig. 21, Fig. 22). This GDNF effect on the DA metobilsm to DOPAC and HVA has been also observed in another study, starting from certain amounts of GDNF (Eslamboli et al., 2005).

The results presented in this study are valid for the specific MPTP mouse model, but may differ in other models. In the absence of a perfect transgenic mouse model for PD that would combine the degeneration of DA synapses and neurons with the cytoplasmatic aggregation of insoluble α-synuclein, the MPTP model still appears to be the preferential model for PD (Tande et al., 2006; Dawson 2002). However, a study using unilateral, striatal AAV-CBA GDNF expression in primates came to similar observations: 14ng GDNF/mg tissue, representing about the double amount of GDNF as in the low titre study (Fig. 5.6), led to a bilateral increase in TH protein levels and activity, which could be restricted ipsilaterally by drastically decreasing the amount of GDNF (Eslamboli et al., 2005). Similarly, unilateral GDNF infusions into the intact midbrain led to profound bilateral changes in DA physiology and decrease in feeding behavior (Hudson et al., 1995).

Moreover, in this study 244pg GDNF/mg SNpc (13-fold increase to background level) and 812pg GDNF/mg *striatum* (25-fold increase to background level) seemed to be enough to deliver a structural and functional protection (Fig. 5.6, 5.16, 5.19, 5.21). Higher amounts of GDNF did not provide an increased protective quality (Fig. 5.6, 5.14, 5.19, 5.21). Interestingly, in an *in vitro* study, although astrocytes transduced with a cytomegalovirus (CMV) promoter secrete ~ 10-fold higher concentrations of GDNF in comparison to cells transduced with the GFAP promoter (already 35-fold increase to background level), a concomitant increase in neuroprotection against 6-OHDA was not observed (Sandhu et al., 2009). This indicates that higher amounts of GDNF are not stringently beneficial since they might also account for side effects (Arvidsson et al., 2003**;** Gill et al., 2003; Zurn et al., 2001; Kordower et al., 1999). However, there seems to exist a threshold under which GDNF is no longer protective (Lindgren et al., 2008; Costa et al., 2001, Zhang et al., 1997). For example, in this study 124pg GDNF/mg *striatum* seemed to be under that threshold (Fig. 5.6, 5.19, 5.21). This concentration-dependent effect of GDNF can be partly explained by opposite

effects of low and high striatal GDNF levels on the TH expression in DA neurons (Kordower et al 2000; Georgievska et al., 2002 a and b).

6.2 The presence of the RET receptor as prerequisite for the protective and regenerative capacity of GDNF

Tyrosine kinases signal through the MAPK and/or the PI3-kinase pathway. For the activation of PI3-kinase/Akt higher concentrations are necessary than for the activation of the MAPK ERK1/2, and only the higher concentration provides protection against MPTP toxicity (Lindgren et al., 2008). GDNF may thus have qualitatively different effects on RET-induced signalling at different concentrations. Therefore, it was decided to use the high titre dosage of $2x10^8$ transducing units producing high amounts of GDNF. Moreover, AAV-5 GFAP GFP or AAV-5 GFAP GDNF were unilaterally injected, thus restricting the effects of GDNF to the injected brain side and being able to use the contralateral side as clear internal control for each mouse.

A conditional RET-deficient mouse model was used and found that RET-dependent signalling mediated through striatal GDNF overexpression protected nigral DA neurons from MPTP-induced degeneration and depletion of catecholamines in the *striatum*. The overexpression of GDNF in mice with RET deficiency did not protect against MPTP toxicity (Fig. 5.31, 3.32). Furthermore, reproducing the results of a previous study in this lab, the recovery of striatal catecholamine levels between two weeks and three months after MPTP intoxication was dependent on the presence of RET (Fig. 5.34). One explanation, why no such a recovery was seen in this study about astrocytic versus neuronal production of GDNF in C57Bl/6J mice (Fig. 5.21, Fig. 5.22) might possibly be that the susceptibility to MPTP depends on MAO-B activity and on the capacity to sequester MPP^+ into synaptic vesicles via the vesicular monoamine transporter (VMAT) as cellular protective mechanism, probably based on the ratio of DAT and VMAT (Miller et al., 1999; Takahashi et al., 1997; Liu et al., 1992). This might be an explanation for mouse strains reacting differently to the toxin.

Striatal recovery of catecholamine levels can generally be due to (i) neurogenesis of DA neurons in the SNpc, outgrowing new axonal connections to the *striatum*; (ii) recovery of axons originating from the somata of injured cells; or (iii) a sprouting response in the terminal field of the nigrostriatal pathway by surviving DA axons. To date no evidence for the neurogenesis of DA neurons after lesioning of the nigrostriatal pathway exists (Frielingsdorf et al., 2004). Further, in a previous study no outgrowth of axons close to the SNpc, but an increased DA fibre density in the *striatum* was shown (Kowsky et al., 2007). Thus the above mentioned third mechanism has to be responsible for the observed striatial restoration of catecholamine levels. In RET deficient mice the overexpression of GDNF was not able to

induce recovery of striatal catecholamine levels three months after MPTP intoxication (Fig. 5.34).

These results are unlikely to be influenced by factors that may alter MPTP toxicity because the following factors did not differ between genotypes: (i) the basal number of DA neurons, the primary density of TH-positive and DAT-positive fibres and the seminal expression of DAT; (ii) the conversion of MPTP to MPP^+; (iii) the glial response (Kowsky et al., 2007); and (iv) the GFP and GDNF expression levels (Fig. 5.24, 5.25, 5.27, 5.28).

NCAM has been proposed as an alternative signalling receptor for GDNF (Paratcha et al., 2003) and RET independent GDNF signalling has been already described (Sariola and Saarma 2003). The results of this study suggest that besides the classical GDNF-mediated activation of RET, no alternative GDNF receptors are involved in the protection and regeneration of the nigrostriatal system against MPTP toxicity in mice. This result can be possibly explained by the expression pattern of GFRα and RET in different brain regions. In many areas of the nervous system, especially in the forebrain, cortex and inner ear GFRα receptors are much more widely expressed than RET (Ylikoski et al., 1999; Kokaia et al., 1999; Trupp et al., 1997), but RET is easily detectable in the murine *striatum* and SNpc (Kramer et al., 2007; Jain et al., 2006) suggesting that GFLs may signal independently of RET in collaboration with other transmembrane proteins only in some areas of the brain. However, although dispensable for the establishment of the nigrostriatal system, the presence of RET is a prerequisite for long-term maintenance and protective/regenerative effects of GDNF to the nigrostriatal system after MPTP lesion (Kramer et al., 2007).

6.3 Perspectives

In this study it was found that GDNF produced by astrocytes is as effective as neuronal GDNF in protecting unilaterally the murine nigrostriatal system against MPTP toxicity, even by using in low quantity GDNF. In the long-term view, even low amounts of neuronal-produced GDNF also protected contralateral structures, what supports the hypothesis that neurons, once they are transduced, release GDNF in all terminal projection areas with uncontrollable effects. In order to further prepare the ground for a gene therapy of PD patients by using astrocytic GDNF expression (AAV-5 GFAP GDNF), a repetition of the experiment with the modification to inject GDNF after MPTP treatment should be performed. Since approximately 60% of the SNpc DA neurons and 80% of their axonal projections in the putamen have been already lost at the onset of PD symptoms (Dauer and Przedborski 2003) a protection via GDNF after MPTP treatment would much better reflect the situation in PD patients.

In order to further elucidate via which tracks astrocytic and neuronal GDNF is transported in the brain AAVs producing fluorescent or mutated GDNF should be injected. These GDNF

variants indeed can indeed be distinguished from endogenous GDNF, but might possibly influence the biological function of GDNF.

Since neurons and astrocytes express different GDNF signalling components, the usage of separate pathways to mediate autocrine and paracrine effects of GDNF could be an explanation for the detected differences in astrocytic or neuronal production of GDNF. Thus, it would be interesting to find out which signalling pathways are active in the presence of astrocytic or neuronal GDNF.

Further, it would be fascinating to distinguish between anterogradely and retrogradely transported GDNF in order to prove the hypothesis about a missing protective effect of anterogradely transported GDNF due to transport-dependent modifications. In this context an experimental setup to discriminate between intra- and extracellular GDNF would be useful.

The measurement of GDNF in the ELISA three months after injection of high and low titres dosages would give the opportunity to include long-term GDNF transportation in the argumentation for astrocytic GDNF having the advantage over neuronal GDNF regarding safety issues for a hypothetical gene therapy in PD patients.

One potential method to address and avoid potential augmented risks in clinical trials might be to utilize a regulated viral vector system, for example the tetracycline (tet)-transcriptional regulation system. The advantages of such an external regulable system would be the possibility to find individual doses, circumvention of unexpected side-effects and the termination of the treatment if no longer effective. In contrast, there is no clinically relevant regulatable vector system currently available. Second, the available gene regulation schemes represent a potential toxicity issue on their own since in all cases, a portion of the regulated system must be constitutively expressed. The third contra-argument is that if no GDNF-induced side-effects can be demonstrated even at the highest possible over-expression levels in pre-clinical toxicology studies, then any requirement to add a potential complication such as regulated vector constructs to the experiment would be unwarranted (Kordower and Olanow 2009, Manfredsson et al., 2009).

The results regarding the RET study revealed that there is no alternative to an intact GDNF-RET mediated signalling in the nigrostriatal system. RET is a prerequisite for the protective potential of GDNF and the natural regenerative capacity of the *striatum* after MPTP lesion in mice. In terms of that study different proof of principle experiments could be performed:

A MPTP experiment with NCAM-deficient mice (Bukalo et al., 2004; Cremer et al., 1994) in combination with AAV-mediated GDNF overexpression should not show disturbances in the protective effect of GDNF to the nigrostriatal system and the natural regenerative capacity of the *striatum*.

Mice with inducible RET expression in the dopaminergic system would provide a tighter connection between the presence of RET and the protective effect of GDNF, if restoration of GDNF-mediated protection could be observed after induction of RET expression.

Furthermore, the question, of whether besides the activation of RET signalling by GDNF other GFLs might also lead to protective and/or regenerative effects still has to be elucidated. A MPTP experiment with GDNF-deficient mice (Pascual et al., 2008; Moore et al., 1996) in combination with AAV-mediated overexpression of other GFLs would demonstrate if other GFLs are also capable to induce protection in the nigrostriatal system or if they are important for the natural regenerative capacity of the *striatum*.

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