GDNF and alpha-synuclein in nigrostriatal degeneration

Maria Chermenina

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Department of Integrative Medical Biology
Section for Histology and Cell Biology
Umeå Universitet, Umeå, Sweden
Cover illustration: Photograph of healthy TH-positive neurons in the substantia nigra.

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Любимой маме посвящается

(To my dear mother)
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## Abbreviations

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<tr>
<td>ALDH1</td>
<td>Aldehyde dehydrogenase 1 A 1</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AP</td>
<td>Anterior-posterior</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<tr>
<td>CT-1</td>
<td>Cardiotropin-1</td>
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<tr>
<td>DARPP-32</td>
<td>Dopamine and cyclic AMP-regulated phosphoprotein of relative molecular mass 32,000</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>DV</td>
<td>Dorso-ventral</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<tr>
<td>GFL</td>
<td>GDNF family of ligands</td>
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<tr>
<td>GIRK2</td>
<td>G-protein activated inwardly rectifying potassium channel</td>
</tr>
<tr>
<td>GFRα1</td>
<td>GDNF-family receptor α1</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>3,4-dihydroxy-L-phenylalanine</td>
</tr>
<tr>
<td>LGE</td>
<td>Lateral ganglionic eminence</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase pathway</td>
</tr>
<tr>
<td>ML</td>
<td>Medio-lateral</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NCAM</td>
<td>Neural cell adhesion</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NT</td>
<td>Neurotrophin</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron-emission tomography</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RARE</td>
<td>Rapid acquisition relaxation</td>
</tr>
<tr>
<td>ROIs</td>
<td>Regions of interest</td>
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<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SNCA</td>
<td>Synuclein Alpha gene</td>
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<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TOM20</td>
<td>Translocase of outer membrane 20</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Voltage-dependent anion channel 1</td>
</tr>
<tr>
<td>VM</td>
<td>Ventral mesencephalon</td>
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Abstract

Parkinson’s disease is a common neurological disorder with a complex etiology. The disease is characterized by a progressive loss of dopaminergic cells in the substantia nigra, which leads to motor function and sometimes cognitive function disabilities. One of the pathological hallmarks in Parkinson’s disease is the cytoplasmic inclusions called Lewy bodies found in the dopamine neurons. The aggregated protein α-synuclein is a main component of Lewy bodies. In view of severe symptoms and the upcoming of problematic side effects that are developed by the current most commonly used treatment in Parkinson’s disease, new treatment strategies need to be elucidated. One such strategy is replacing the lost dopamine neurons with new dopamine-rich tissue. To improve survival of the implanted neurons, neurotrophic factors have been used. Glial cell line-derived neurotrophic factor (GDNF), which was discovered in 1993, improves survival of ventral mesencephalic dopamine neurons and enhances dopamine nerve fiber formation according to several studies. Thus, GDNF can be used to improve dopamine-rich graft outgrowth into the host brain as well as inducing sprouting from endogenous remaining nerve fibers. This study was performed on Gdnf gene-deleted mice to investigate the role of GDNF on the nigrostriatal dopamine system. The transplantation technique was used to create a nigrostriatal microcircuit from ventral mesencephalon (VM) and the lateral ganglionic eminence (LGE) from different Gdnf gene-deleted mice. The tissue was grafted into the lateral ventricle of wildtype mice. The results revealed that reduced concentrations of GDNF, as a consequence from the Gdnf gene deletion, had effects on survival of dopamine neurons and the dopamine innervation of the nigrostriatal microcircuit. All transplants had survived at 3 months independently of Gdnf genotype, however, the grafts derived from Gdnf gene-deleted tissue had died at 6 months. Transplants with partial Gdnf gene deletion survived up to 12 months after transplantation. Moreover, the dopaminergic innervation of striatal co-grafts was impaired in Gdnf gene-
deleted tissue. These results highlight the role of GDNF for long-term maintenance of the nigrostriatal dopamine system. To further investigate the role of GDNF expression on survival and organization of the nigrostriatal dopamine system, VM and LGE as single or combined to double co-grafts created from mismatches in Gdnf genotypes were transplanted into the lateral ventricle of wildtype mice. Survival of the single grafts was monitored over one year using a 9.4T MR scanner. The size of single LGE transplants was significantly reduced by the lack of GDNF already at 2 weeks postgrafting while the size of single VM was maintained over time, independently of GDNF expression. The double grafts were evaluated at 2 months, and the results revealed that lack of GDNF in LGE reduced the dopamine cell survival, while no loss of dopamine neurons was found in VM single grafts. The dopaminergic innervation of LGE was affected by absence of GDNF, which also caused a disorganization of the striatal portion of the co-grafts. Small, cytoplasmic inclusions were frequently found in the dopamine neurons in grafts lacking GDNF expression. These inclusions were not possible to classify as Lewy bodies by immunohistochemistry and the presence of phospho-α-synuclein and ubiquitin; however, mitochondrial dysfunction could not be excluded. To further study the death of the dopamine neurons by the deprivation of GDNF, the attention was turned to how Lewy bodies are developed. With respect to the high levels of α-synuclein that was found in the striatum, this area was selected as a target to inject the small molecule – FN075, which stimulates α-synuclein aggregation, to further investigate the role of α-synuclein in the formation of cytoplasmic inclusions. The results revealed that cytoplasmic inclusions, similar to those found in the grafts, was present at 1 month after the injection, while impairment in sensorimotor function was exhibited, the number of dopamine neurons was not changed at 6 months after the injection. Injecting the templator to the substantia nigra, however, significantly reduced the number of TH-positive neurons at 3 months after injection. In conclusion, these studies elucidate the role of GDNF for maintenance and survival of the nigrostriatal dopamine system and mechanisms of dopamine cell death using small molecules that template the α-synuclein aggregation.
Original papers

This thesis is based on the following papers, which are referred in the text by their roman numerals:


*Equal contribution


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Introduction

Parkinson's disease

Parkinson’s disease is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta, which project their axons to the striatum, leading to reduction in dopamine levels in the entire basal ganglia (Bernheimer et al., 1973; Ehringer and Hornykiewicz, 1960; Trétiakoff, 1919). The histopathological hallmark of Parkinson’s disease is the appearance of cytoplasmatic inclusion bodies in the dopamine neurons, called Lewy bodies, which contain the aggregated protein α-synuclein (Lewy, 1912; Spillantini et al., 1998). Parkinson’s disease involves also degeneration of non-dopaminergic cells of the nervous system such as serotonergic, noradrenergic, and cholinergic neurons, including the spinal cord and the peripheral autonomic nervous system (Bloch et al., 2006; Kish et al., 2008; Nakano and Hirano, 1984; Zarow et al., 2003). The cause of the disease is still unknown, however, mutations in several genes such as ubiquitin, parkin, or α-synuclein (SNCA gene) were pointed out as possible explanations for Parkinson’s disease pathogenesis (Lotharius et al., 2002; Shimura et al., 2000). Other possible causes of Parkinson’s disease, discussed during recent years, are mitochondrial dysfunction, leading to free radical release and oxidative damage of dopamine neurons as well as a prion-like disease theory, when Parkinson’s disease pathology is claimed to start in the enteric nervous system to further propagate to the brain stem via the vagus nerve (Braak et al., 2004; Braak et al., 2006; Schapira et al., 1989).

All established therapies for Parkinson’s disease patients are focused on relieving the symptoms. Thus, no curable treatment is available to date. Current treatments include drug treatments such as L-DOPA, dopamine agonists, monoamine oxidase B (MAO) or the catechol-O-methyl transferase (COMT), which all are aimed to increase striatal dopamine levels however, these treatments give rise to side effects, such as efficacy decline with time, development of dyskinesias and on-off symptoms (Birkmayer and Hornykiewicz,
1962; Carlsson et al., 1957; Granerus, 1978; Lew et al., 2007; Pellicano et al., 2009; Rinne, 1981). Nondrug treatment, such as deep brain stimulation, which consists of an electrode inserted into the subthalamic nucleus, the globus pallidus or the thalamus is a potent treatment to reduce Parkinson's disease symptoms, though is usually used at late stages of the disorder when medical drugs are less efficient to relieve from symptoms (Benabid et al., 1991; Benabid et al., 1998; Kumar et al., 1998). Therefore, the discovery of novel drugs and treatment strategies is of great importance. Neurotrophics factors, grafting of fetal tissue, or using small molecules to modulate α-synuclein aggregation are possible future treatment strategies.

Transplantation in Parkinson's disease

Transplantation of neuronal tissue is a nondrug method in attempts to increase the dopamine levels and restore the number of dopamine neurons in the brain of Parkinson's disease patients. The history of neuronal tissue transplantation began in early 1970s, when adrenal medulla and fetal nigral cells were successfully transplanted into the anterior eye chamber, which was followed by transplantation of fetal nigral cells into the rat brain (Olson and Malmfors, 1970; Olson and Seiger, 1972; Stenevi et al., 1976). Since then, many studies have been performed on dopaminergic transplantation in animal models of Parkinson’s disease beginning in the late 1970s. It was demonstrated that fetal dopaminergic grafts could survive and produce axonal outgrowth into the host brain and reduction of motor abnormalities occurred in the rodent model of Parkinson’s disease (Bjorklund and Stenevi, 1979; Dunnett et al., 1981; Perlow et al, 1979). In 1980, the method of cell suspension transplantation was established in animals (Bjorklund et al., 1980). Thereafter, it was shown that nigral grafts not only survived for long-term time periods but also could functionally reactivate the deafferented striatum and form new dopamine synapses including dopamine release (Bjorklund et al., 1981; Bolam et al., 1987; Freed et al., 1980; Freund et al., 1985; Jaeger, 1985; Mahalik et al., 1985; Rose et al., 1985; Stromberg et al., 1988; Stromberg et al., 1992; Stromberg and Bickford, 1996; Zetterström et al., 1986).
One obstacle with transplantation to Parkinson’s disease patients is the poor survival of grafted dopamine neurons: approximately 10% of transplanted dopaminergic cells survive the grafting procedure (Barker et al., 1996; Sortwell et al., 2000). One possible explanation for the poor survival might be graft placement in the brain. Two target regions for grafting with their advantages and disadvantages, either the striatum or the ventral mesencephalon (VM) have been selected in most animal studies (Herman et al., 1991; Nikkhah et al., 1995a; Nikkhah et al., 1995b). Placement of fetal nigral grafts into the striatum, which is the target area for projections from the nigral cells, may cause incomplete reinnervation and recovery due to lack of specific physiological environmental factors for growth, maintence and survival. On the other side, the dopamine neurons, placed in homotopic ontogenic site i.e. in the substantia nigra, are not capable to project their axons over the long distance to reach their striatal target (Dunnett et al., 1989; Schnell and Schwab, 1990).

Encouraging results from animal studies led to the first clinical trials, which were conducted in 1982, when adrenal medullary grafts were grafted in patients with Parkinson’s disease (Backlund et al., 1985). Later, several reports demonstrating transplantation of fetal nigral grafts in Parkinson’s disease patients with evidence of graft survival and functional recovery were reported (Freed et al., 1995; Freeman et al., 1995; Kordower et al., 1995; Lindvall et al., 1988; Lindvall et al., 1989; Lindvall et al., 1990; Madrazo et al., 1988). Evidence of regulated dopamine release from nigral grafts and graft-induced restoration of movement-related cortical activation in Parkinson’s disease patients was proved utilizing positron-emission tomography (PET) (Piccini et al., 1999; Piccini et al., 2000). Further results from two double blind, placebo-controlled grafting trials of fetal ventral mesencephalon were published in 2001 and 2003 revealed no significant clinical improvement in patients with Parkinson’s disease. Moreover, the phenomenon of graft-induced dyskinesia was reported for the first time, which may be explained by an aberrant synaptic plasticity of the host medium-sized spiny neurons innervated by the dopamine transplants (Freed et al., 2001; Olanow et al., 2003; Rylander, 2013). Usefulness of neuronal transplantation has frequently been discussed in recent years (Barker et al.,
Due to the inter-individual variability of open-label studies and the occurrence of post-transplantation dyskinesia, a new multicenter trial TRANSEURO, led by Dr. Roger Barker, sponsored by the European Union started in 2010. The aim of this project is to reanalyze fetal cell based treatment using the step-by-step optimization of the delivery of fetal dopaminergic midbrain grafts for Parkinson’s disease patients under more controlled and centralized conditions. The project is still under patient recruiting stage (http://transeuro.org.uk).

One positive finding is the long-term survival of the transplants. However in two clinical trials, postmortem evaluations of dopaminergic transplants demonstrated brain pathology, typical for Parkinson’s disease thus, α-synuclein-positive inclusions (Lewy bodies) (Kordower et al., 2008; Li et al., 2008). In a third study demonstrating long-term graft survival, no Lewy bodies could be demonstrated in the transplants (Mendez et al., 2008). However, the fact that α-synuclein inclusions had been found, raised a debate whether Parkinson’s disease is of prion-like nature (Ahlskog, 2007; Braak and Del Tredici, 2008; Lang and Obeso, 2004; Langston, 2006).

The progress in transplantation using human embryonic tissue brought complex logistic, ethical, and legal issues needed to be considered and followed (Boer, 1994). To avoid these issues, the use of human embryonic stem cells (hESC), induced pluripotent stem cells (iPSC), induced neuronal cells (iN cells), and induced dopaminergic cells (iDA cells) was proposed (Caiazzo et al., 2011; Cho et al., 2008; Pfisterer et al., 2011; Rosser et al., 2007; Takahashi et al., 2007; Vierbuchen et al., 2010; Wernig et al., 2008). Despite promising results from recent studies using stem cells, additional work is needed to clarify safety issues and to investigate immunogenicity, cell proliferation, and tumor formation (Barker and Widner, 2004; Hou et al., 2013; Kikuchi et al., 2011; Kirkeby et al., 2012; Kriks et al., 2011; Pang et al., 2011; Wernig et al., 2008).

To enhance the survival and functional properties of grafted dopamine cells the combination of transplantation therapy with neurotrophic factors was established in animal studies. Several studies have been performed where the glial cell line-derived neurotrophic factor (GDNF), brain-derived
neurotrophic factor (BDNF), fibroblast growth factor, and netrin-1 have been used to create growth support to transplanted cells with improvement in motor behavior (Brecknell et al., 1996; Wang et al., 1996; White et al., 1999; Wilby et al., 1999; Zhang et al., 2013). However, further investigations are still needed to reach clinical relevant behavioral improvement.

**Neurotrophic factors**

Neurotrophic factors have been proposed as a possible treatment for patients suffering from Parkinson's disease to rescue the dopamine neurons from cell death and to induce sprouting. The first nerve growth factor (NGF) was discovered by Rita Levi-Montalcini in 1951 (Cohen and Levi-Montalcini, 1957; Levi-Montalcini and Hamburger, 1951). Since then, discovery, characterization, and studies of trophic factors for their therapeutic effects in the nervous system have been the focus for many scientists. Most of the neurotrophic factors that were discovered under last decades belong usually to one superfamily of neurotrophic factors such as nerve growth factor (NGF)-family, GDNF-family, neurokine or neuropoetin family, and non-neuronal growth factor-family. (Balogh et al., 1998; Barde et al., 1982; Kotzbauer et al., 1996; Lin et al., 1993; Lindholm et al., 2007; Lindholm and Saarma, 2010; Milbrandt et al., 1998; Palgi et al., 2009; Petrova et al., 2003). All these super-families consist of structurally (homology of receptors) and functionally (common transduction pathways) related neurotrophic factors. NGF-family with 4 factors: NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) was the first growth factor family to be identified (Barde et al., 1982; Berkemeier et al., 1991; Cohen and Levi-Montalcini, 1957; Ip et al., 1992; Levi-Montalcini and Hamburger, 1951; Maisonpierre et al., 1990; Rosenthal et al., 1990). The main function of NGF is supporting the survival and differentiation of cholinegic neurons in the central nervous system and sympathetic and sensory neurons in the peripheral nervous system (Date et al., 1997; Ebendal, 1989; Silani et al., 1990; Stromberg and Ebendal, 1989). GDNF family of ligands (GFL) exerts its functions on several different neuronal populations in both the central and the peripheral nervous system with the very important ability to
promote the growth and survival of midbrain dopaminergic neurons (Lin et al., 1993). This family includes 4 known members: GDNF, neurturin, artemin and persephin (Baloh et al., 1998; Kotzbauer et al., 1996; Lin et al., 1993; Milbrandt et al., 1998). Other neurotrophic factors such as ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), cardiotropin-1 (CT-1) and oncostatin-M are included in the neurokine superfamily with the functions of neuronal and glial differentiation and development (Akira, 1997; Ip and Yancopoulos, 1992; Murphy et al., 1997). Non-neuronal growth factor family has also shown neurotrophic effects and can enhance dopamine fiber formation from nigral grafts (Giacobini et al., 1993). This family includes acidic and basic fibroblast growth factors (FGF-1 and FGF-2), epidermal growth factor (EGF), insulin-like growth factor (IGF) and bone morphogenic protein (BMP) (Gospodarowicz et al., 1978). Participation of neurotrophic factors in such functions as axonal growth and neuronal development, survival, and modulation have possibly a great therapeutic value for Parkinson's disease and for many other degenerative disorders as well as after injury of the nervous system.

**GDNF**

In 1993, GDNF, isolated from rat B49 glial cell-line supernatant by Lin and colleges, was shown to enhance effects on dopaminergic neurons in terms of neuronal survival and morphological differentiation. Since that many studies were established to reveal distribution and mechanisms of function of GDNF in the nervous system (Eggert et al., 1999; Kirik et al., 2001; Lin et al., 1993). GDNF is a small extracellular peptide and belongs to GFL, which is related to the transforming growth factor superfamily (Lin et al., 1993). GDNF is initially synthesized as 211 amino acid long preproGDNF and then becomes proGDNF by being cleaved during secretion to the lumen of endoplasmic reticulum followed by the transport to the Golgi apparatus to become the 134 amino acid long mature homodimeric active form of 32-42 kDa. (Boado et al., 2008; Cristina et al., 1995; Grimm et al., 1998; Ibanez, 1998). GDNF acts via a heterodimeric receptor tyrosine kinase (Ret) and the ligand binding component
GDNF-family receptor α1 (GRFα1) (Jing et al., 1996; Treanor et al., 1996; Worby et al., 1996). Upon GDNF binding to the receptors the decision about cell survival or death proceeds through the intracellular phosphoinositol 3 kinase signaling and the mitogen-activated protein kinase pathway (Ras-MAPK) (Fig. 1) (Nicole et al., 2001; Worby et al., 1996). Two more intracellular pathways have been reported: Jun N-terminal kinase and PLCγ-dependent pathways that can be triggered by Ret (Borrello et al., 1996; van Weering and Bos, 1998). GDNF can also signal independently of Ret via neural cell adhesion molecule (NCAM) or GRFα2, which normally is the primary receptor for neurturin (Paratcha et al., 2003; Sanicola et al., 1997; Trupp et al., 1997; Trupp et al., 1999).

**Figure 1.**
A simplified schematic drawing showing GDNF signaling pathway. GDNF exert neurotrophic actions via GRFα1 and ret receptors binding a heterocomplex on the membrane of the neuron. The major signaling pathways are MAPK and PI3K.

GDNF mRNA is detectable in several structures of the nervous system such as the striatum, hippocampus, cortex, cerebellum, and spinal cord. The levels of GDNF mRNA are higher in the developing brain than in the adult brain regions, which indicates the important role of GDNF during brain development.
(Springer et al., 1994; Stromberg et al., 1993). Moreover, GDNF mRNA expression was reported in peripheral tissues, for instance in kidney and testis (Yamamoto et al., 1996).

**GDNF effects on dopamine neurons**

Initially, in vitro studies demonstrated increased survival and decreased apoptosis of dopamine neurons in VM cultures from rat, monkey, and human (Clarkson et al., 1997; Kaddis et al., 1996; Krieglstein et al., 1995; Lin et al., 1993; Meyer et al., 2000). Moreover, GDNF protects against toxins, usually used to produce animal models of Parkinson’s disease, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Beck et al., 1995; Eggert et al., 1999; Hou et al., 1996; Tomac et al., 1995a). These results gave rise to numerous in vivo studies using direct bolus injection of GDNF into the striatum, lateral ventricle or the substantia nigra. In vivo studies confirmed the protection properties of GDNF on dopamine neurons when injected directly into the striatum, the substantia nigra or to the region just above the substantia nigra at 1-week after 6-OHDA lesions (Kearns and Gash, 1995; Sauer et al., 1995). Later it was demonstrated that one single bolus injection of GDNF into the striatum of dopamine-lesioned animals not only protected dopamine neurons from dying but also preserved the striatal tyrosine hydroxylase (TH) levels, which indicated a preservation of motor function (Kirik et al., 2000). The intraventricular administration of GDNF in rodents demonstrated GDNF diffusion from cerebrospinal fluid into superficial as well as deep brain structures resulting in increased levels of striatal and nigral dopamine (Lapchak et al., 1997; Martin et al., 1996). The intraventricular injections of GDNF in non-human primates did not shown the same optimistic results as in rodent models. In this case, GDNF did not appear to diffuse easily into the striatum (Lapchak et al., 1998). However, intrastriatal delivery of GDNF in non-human primate gave promising results in terms of improved dopamine neuron survival and motor functions (Grondin et al., 2002; Maswood et al., 2002). However, recent studies reported toxicity in terms on
multifocal cerebellar Purkinje cell loss after six months of chronic infusion of GDNF into the putamen of primates (Hovland et al., 2007).

**GDNF-deficient mice**

Most studies on GDNF concerned addition of GDNF to cell cultures or animal models, but what should happen to the nigrostriatal system in the totally absence of GDNF? Gdnf gene-deleted mouse was presented as an animal model to study neuroprotection in 1996 (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). These mice totally lack the enteric nervous system and the kidneys and died therefore shortly after birth. Due to the premature death, the Gdnf gene-deleted tissue can only be studied in situ during fetal stages. No differences in distribution, density, number and size of dopamine and locus ceruleus noradrenergic neurons were found in Gdnf gene-deleted mice at birth compared to normal mice (Moore et al., 1996). However, slice cultures from embryonic day 14 tissue revealed that the absence of GDNF inhibited neurite outgrowth without affecting neuronal survival (af Bjerken et al., 2007). Nevertheless, mice with one allele of gndf gene (heterozygous) are viable but their dopamine system decline with age with the consequence of motor dysfunctions (Airavaara et al., 2004; Boger et al., 2006).

**GDNF in clinical trials**

The first randomized double-blind placebo-controlled study was performed by Nutt and colleges in 2003 and demonstrated no clinical improvement after intracerebroventricular infusion of GDNF. Moreover, patients expressed side effects in form of weight loss, nausea, anorexia, and vomiting (Nutt et al., 2003). The authors explained their findings with inadequate diffusion of GDNF into the striatum and the substantia nigra in the human brain. Delivery of GDNF into the brain continued and two open-label studies of continuous intraputamenal infusion of GDNF via microcatheters attached to an infusion pump demonstrated improvements in all clinical parameters as well as increase in density of TH-positive nerve fibers (Gill et al., 2003; Love et al., 2005; Patel et al., 2005; Slevin et al., 2005). However, a
multicenter randomized controlled trial of intraputamenal GDNF infusion to patients with Parkinson’s disease demonstrated no clinical improvements. Moreover, neutralizing antibodies against GDNF were found in some of the patients (Lang et al., 2006). The failure of this study was explained by technical variations in catheter delivery and design of studies, as well as poor bioavailability of GDNF due to poor diffusion (Salvatore et al., 2006).

GDNF delivery utilizing virally-mediated gene therapies revealed a significant potential in primates and therefore might be a possible effective method to solve the delivery problems (Johnston et al., 2009; Kells et al., 2010; Su et al., 2009). Though, clinical studies showed no significantly benefits from GDNF delivered by viral vector (Bartus et al., 2007). A possibly explanation might be insufficient retrograde transport from the striatum (the site for infusion) to the substantia nigra (De Vos et al., 2008; Roy et al., 2005).

The powerful trophic actions of GDNF on dopamine neurons were documented by many studies since 1991. However, the clinical studies have yet not confirmed the positive effects of GDNF in patients with Parkinson’s disease, and therefore existing methods of treatment or delivery of GDNF need to be explored.

Ethiopathogenesis of Parkinson’s disease

Mitochondrial dysfunction

There is no doubt that mitochondria are essential for cellular function and in particular for neurons with their large energy requirements. Mitochondria have a critical role in ATP production, calcium homeostasis, and apoptotic processes (McBride et al., 2006). They produce energy in the form of ATP by oxidative phosphorylation via the electron transport chain, which is composed of five multiprotein complexes, I-V (Saraste, 1999). Involvement of mitochondria in oxidative stress and therefore susceptibility to oxidative damage was described in the early 1990s (Richter and Kass, 1991; Sohal and Brunk, 1992). To date, impaired activity of mitochondrial complex I have been claimed to be associated with the pathogenesis of Parkinson’s disease (Di
Monte et al., 1992). This was described for the first time after an observation of acute parkinsonian syndrome in some drug abusers that accidentally had tested MPTP, which is an inhibitor of mitochondrial complex I (Langston et al., 1983). Subsequently, it was shown that MPTP selectively destroys dopamine neurons in the substantia nigra pars compacta and therefore this drug begun to be used to produce an animal model for Parkinson’s disease (Burns et al., 1983; Hallman et al., 1984; Sundstrom et al., 1987). A reduction of complex I activity was reported in the brain of patients with Parkinson’s disease (Parker et al., 1989; Schapira et al., 1990).

Mitochondria constantly undergo dynamic cycles of fusion and fission to maintain its function (Detmer and Chan, 2007; Knott et al., 2008). It has been shown that cells require mitochondrial fusion for proper respiratory activity (Chen et al., 2007). On the other hand, neuronal death is associated with mitochondrial fission (Barsoum et al., 2006; Meuer et al., 2007). It was noted that α-synuclein causes mitochondrial fragmentation when it binds to mitochondrial membranes (Kamp et al., 2010; Nakamura et al., 2011). In addition, the autophagic degradation of mitochondria seems to be impaired in Parkinson’s disease, which leads to accumulation of abnormal mitochondria in the cells, which in turn might contribute to cell death (Vila and Przedborski, 2003). However, it is unknown if mitochondrial alteration in Parkinson’s disease is a primary event or a consequence of other factors contributing to the pathogenesis of Parkinson’s disease. Thus, more studies are needed to investigate this issue.

**Alpha-synuclein**

The protein synuclein was named due to its localization to the nuclear envelope of neurons and to presynaptic nerve terminals where it was first isolated from *Torpedo californica* in 1998. Alpha-synuclein is 140 amino acid long protein and belongs to the protein family of synucleins (Maroteaux et al., 1988). The primary sequence of α-synuclein consists of 3 structural and functional different regions: the N-terminal region responsible for membrane binding, the non-amyloid-β component amyloid, which nucleates amyloid
formation, and the C-terminal region that is typical for an intrinsically disordered region (Lorenzen et al., 2014). Alpha-synuclein is highly expressed in the brain but is also localized in other tissues, for instance in red blood cells (Jakes et al., 1994; Nakai et al., 2007). The localization of α-synuclein is mainly in cell cytosol and changes during development of the central nervous system from the cell body of neuronal precursors to appear at nerve terminals at adult stages (Hsu et al., 1998; Tobe et al., 1992). Nevertheless, α-synuclein is also found in cerebrospinal fluid and blood plasma, which confirms that α-synuclein can be secreted endogenously (El-Agnaf et al., 2003; Tokuda et al., 2006). Both monomeric and aggregated forms of α-synuclein can be secreted by non-classical exocytotic or endocytotic pathways (Lee et al., 2005). There are many speculations about the functions of α-synuclein, and recent studies suggest that α-synuclein might have a role as a regulatory component of the vesicular transport processes, and synaptic vesicle release and recycling (Davidson et al., 1998; Jenco et al., 1998). Furthermore, α-synuclein has a role in neurotransmitter release such as dopamine and glutamate (Gureviciene et al., 2007; Liu et al., 2004; Yavich et al., 2004).

As mentioned above, Lewy bodies are a major hallmark of Parkinson’s disease. The main content of Lewy bodies is α-synuclein in an insoluble and fibrillar form that is ubiquitinated (Fujiwara et al., 2002a; Spillantini et al., 1998). In addition, approximately 15% of α-synuclein in the Lewy bodies is C-terminally truncated and 90% is phosphorylated (Bisaglia et al., 2009; Fujiwara et al., 2002b). The highly charged C-terminal region has been proposed to protect α-synuclein from polymerization (Levitan et al., 2011). There is evidence that inhibition of α-synuclein aggregation, which is formed from monomers via dimers and oligomers to aggregates, may be associated with a decrease of α-synuclein toxicity. This means that formation of Lewy bodies including the fibrilar form of α-synuclein is a defense mechanism against the toxic soluble oligomeric α-synuclein (Conway et al., 2000; Periquet et al., 2007; Winner et al., 2011). Thus, appearance of extracellular α-synuclein and inter-neuronal transmission of α-synuclein seems to match the progressive
development of Parkinson’s disease and further strengthens the prion-like nature of Parkinson’s disease (Braak et al., 2003; Desplats et al., 2009).

**Small molecules**

Many environmental factors such as heavy metals and pesticides may promote α-synuclein aggregation (Uversky et al., 2001a; Uversky et al., 2001b). Therefore, several studies have been initiated to find small chemical molecules that can modulate α-synuclein aggregation in an attempt to elucidate the molecular mechanism and biological consequences of α-synuclein aggregation and to find diagnostics and novel treatment methods for patients suffering from Parkinson’s disease. Conmay and colleagues screened 169 different compounds to find potential inhibitors of α-synuclein fibrillary formation. They published 15 potential inhibitors, most of them were catecholamines (dopamine, L-dopa, epinephrine and norepinephrine) that had inhibitory activity of α-synuclein fibrillary formation (Conway et al., 2001). It was also found that the antibiotic rifampicin inhibited α-synuclein aggregation, which resulted in decreased neurotoxicity (Li et al., 2004). Moreover, it has been reported that the mechanism of the anti-parkinsonian drug selegiline forms nontoxic aggregates of α-synuclein via a delay of nucleation phase, and the flavonoid baicalein inhibits fibrillation of α-synuclein by induction of spherical α-synuclein oligomer production that cannot proceed to fiber formation (Braga et al., 2011; Hong et al., 2008).

It is speculated that small natural peptides may modulate α-synuclein aggregation (Fonteh et al., 2007; Lewitt et al., 2013; Lindersson et al., 2005). It was recently demonstrated that a small molecule with a dihydro-thiazolo ring-fused 2-pyridone with a central fragment, designed to mimic a small C-terminal peptide, named FN075, promotes aggregation of α-synuclein (Horvath et al., 2012). FN075 exerts inhibiting effects on the Alzheimer β-peptide aggregation (Aberg et al., 2005). It was demonstrated that variation in compound substitutions results in opposite effect on fiber aggregation (Akaishi et al., 2008). Therefore, small chemical modification of the 2-pyridone containing central fragment may result in compounds that inhibits α-synuclein
aggregation (Horvath et al., 2013). Thus, to elucidate the effects of these small molecules might be important to understand the toxicity of α-synuclein aggregation in Parkinson’s disease.
Aims of the thesis

➢ To study the VM dopamine neuronal dependency on GDNF

➢ To examine the importance of GDNF for organization of the striatal anlage

➢ To investigate the effects of small molecules (dihydro thiazolo ring-fused 2-pyridones) on the nigrostriatal dopamine system with respect to templation of endogenous α-synuclein
Materials and methods

Animals

All animal protocols have been approved by the local ethics committee and carried out according to international guidelines on the protection of animals used for scientific purposes of the European Parliament and of the Council Directive (2010/63/EU).

Gdnf-/- mice on a C57Bl/6J background (Pichel et al., 1996) were used in the grafting studies (Papers I and II). Female C57Bl/6 mice (8 weeks) were purchased from Charles River (Sulzfeld, Germany) to inject the small molecules (modified 2-pyridone variants) affecting α-synuclein oligomerization into the striatum and the substantia nigra (Paper III). All animals were kept in standardized conditions with 12:12 artificial light-dark cycle, constant ambient temperature and were fed with standard rodent pellets and tap water ad libitum. All efforts were made to minimize the number of animals used in the experiments and their suffering.

Intracranial transplantation

In papers I and II, Gdnf +/- and Gdnf +/+ mice were mated over the same night for combining different genotypes of the ventral mesencephalon (VM) and the lateral ganglionic eminence (LGE) to transplant as single grafts or to create the nigrostriatal microcircuit with different Gdnf genotype combinations. Embryonic day 14 (E14) fetuses were collected from deeply anesthetized and neck dislocated pregnant mice (4% isofluran, Baxter Medical AB, Kista, Sweden). Tissue pieces of fetal VM and LGE were aseptically dissected and placed in sterile Dulbecco’s modified Eagle medium 1X (DMEM; Gibco, Grand Island, USA). In order to genotype, fetal tail samples were stored at -30°C until the genotype procedure. The transplants (single or double) were implanted stereotaxically into the right ventricle of isofluran anesthetized 3-5-month-old female Gdnf +/- mice at the following coordinates: anterio-posterior (AP) at bregma level, medio-lateral (ML) -0.8 mm from bregma, dorso-ventral (DV) -3.5 mm from the dura mater (Fig. 2).
Figure 2.
Schematic drawing of experimental designs for papers I and II. To obtain embryos of all Gdnf genotypes, Gdnf +/- mice were mated over one night. Embryos were collected at embryonic day (E) 14 and VM and LGE regions were dissected to be transplanted into the right lateral ventricle of wildtype mice in 4 different combinations as shown in A, B, C, and D.
Compounds to affect α-synuclein aggregation

Compounds, FN075 (the templator) and MS382 (the inhibitor), which are 2 pyridones-flat aromatic molecules with peptide-like stretch were synthesized and characterized by our collaborators at the Department of Chemistry, Chemical Biological Center, Umeå University. It has been shown that FN075 stimulates and MS382 inhibits α-synuclein oligomer formation (Horvath et al., 2012; Horvath et al., 2013).

Intracranial injection

In paper III, 8 week-old C57B1/6 mice received intracranial injection of the modified 2-pyridone variants FN075 (the templator) and MS382 (the inhibitor). The compounds were injected either into the striatum or the substantia nigra. Mice injected into the striatum were divided in 3 subgroups: 1) treatment with the templator, animals received 2 µl 1mM FN075 in 10% DMSO in 0.9% NaCl, 2) treatment with the inhibitor, mice received 2 µl 1mM MS382 in 10% DMSO in 0.9% NaCl, and 3) control group, animals received vehicle 2 µl 10% DMSO in 0.9% NaCl. Mice injected into the substantia nigra received either the templator or vehicle. Prior to the injection of the molecules, the animals were anesthetized with isoflurane (Baxter Medical AB, Kista, Sweden), placed in a stereotaxic frame, and a hole was drilled in the skull at the followed coordinates: for the striatal injection: - AP +0.12 mm, ML -0.17 mm from bregma, DV from the dura mater -0.30 mm and for the substantia nigra injection: - AP -0.24 mm, ML -0.14 mm from bregma, DV -0.45 mm from the dura mater. The injections were performed at the rate of 1 µl/2 min by 10 µl syringe (701N, 26s/2”/3; Hamilton Company, Nevada, USA) which was withdrawn 2 min after the injection in order to avoid reflux.

In vivo magnetic resonance imaging (MRI)

Transplanted animals (Paper II) were examined on a 9.4 T horizontal bore MR system (BioSpec 94/20; Bruker, Ettlingen, Germany) with a B-GA12 gradient (450 mT/m) in order to monitor the single transplant size and survival. Acquisition was performed using Paravision 5.1 software. Mice were
anesthetized with isoflurane in O₂ during the entire procedure and breathing rate and body temperature were constantly monitored using Monitoring and Gating System with a rectal temperature probe and a pneumatic pillow (SA Instruments, Inc; Stony Brook, USA). MRI procedure was performed using a 23 mm quadrature transmit receive coil (Bruker). The RARE (Rapid acquisition relaxation enhanced) sequence (Hennig et al., 1986) was used to collect axial/coronal T₂-weighted images with the following parameters: echo time (TE) 11 ms (effective TE 33 ms); repetition time (TR) 2500 ms; number of slices 13-15, slice thickness 0.20 mm; field of view 20 x 20 mm; matrix size 256 x 256; rare factor 8. 16/8 acquisitions were summed following 2 dummy scans.

Images were analyzed in order to calculate the volume of the transplants using Paravision specific processing tools. Regions of interest (ROIs) were created by manually outlining the transplants. The volume of the transplants was calculated from the ROI areas and the slice thickness. The volume is expressed as mean value from coronal and axial calculations.

**Behavioral analysis**

In paper III, the behavioral tests were performed to analyze motor, sensorimotor, motor coordination and balance in mice at 6 months after striatal injection of the templator, the inhibitor, and the vehicle.

**Adhesive removal test**

Sensorimotor functions were tested by placing an adhesive stimulus in form of a quarter of circle of 10 mm in diameter on the snout of the mouse. Two parameters were measured such as the time to sense and the time to remove the sticker from the snout and the removal minus contact time was calculated. All tests were performed in the animal home cage after all cage mates had been moved to another cage. Each mouse received in total 3 trials, which were performed at 3 different days. The average of those trials were taken to calculate the results (Fleming et al., 2004; Schaar et al., 2010; Schallert et al., 1982).
**Cylinder test**

A transparent cylinder, 15 cm in diameter and 12 cm in height, was used to measure spontaneous activity in mice (Fleming et al., 2004; Fleming and Chesselet, 2006; Hwang et al., 2005). To be able to videotape mice movements, the cylinder was placed on the glass with mirror underneath. Each animal was recorded for 3 min and the number of rears (a vertical movements with both forelimb off the bottom of cylinder), the grooming time (bouts of the snout, vibrissae and body), forelimb and hindlimb were counted in slow motion. Values were presented as means ± SEM.

**Pole test**

Motor coordination and balance was evaluated by the pole test (Fleming et al., 2004; Matsuura et al., 1997). The mice were gently placed on the top of the 50 cm long and 1 cm in diameter, vertical, wooden pole with head-upward direction. All mice were trained 2 days before the test day with 5 training trials each day. On the test day, each animal made 3 trials and the time to orient downward and descend the pole was recorded. Statistical evaluations were made on averages of the 3 measurements for each animal.

**Genotyping**

Genotypes of all brain tissue donors were identified by polymerase chain reaction (PCR) of tail biopsy. The tissue was homogenized in 500 μl of 50 mM NaOH in a thermomixer (Thermomixer Compact, Eppendorf, Hornsholm, Denmark) during 15 min shaking at 95°C. The samples were then removed from the thermomixer and neutralized with 50 μl of 1 M Tris-HCl, pH = 7.5. Each PCR sample was prepared by added 1.8 μl dH2O, 5 μl of REDExtract-N-Amp™ PCR (Ready Mix™, Sigma, Stockholm, Sweden), 0.3 μl sense primer and 0.3 μl of antisense primer (Promega, Nacka, Sweden) and 2 μl of DNA sample or dH2O for negative controls. Sense primers, wt – 5´- CCA GAG AAT TCC AGA GGG AAA GGT C – 3’ or knockout – 5´- CGG AGC CGG TTG GCG CTA CGG G – 3’; antisense primer, wt – 5´- CAG ATA CAT CCA CAC CGT TTA CGG G – 3’ or knockout – 5´- ACG ACT CGG ACC GCC ATC GGT G – 3’ were used to detect the Gdnf genes.
termal cycler (PTC-200, MJ Reserch, Inc., Waltham, MA, USA) were used to PCR amplification with 35 amplification cycles in total with the initialization step at 94°C for 3 min followed by 34 cycles, where each cycle included 15 sec denaturation at 94°C, 15 sec annealing at 56°C, and 15 sec elongation at 72°C. Thereafter, the gel electrophoresis was performed to detect Gdnf +/+ allele at 344 basepair and Gdnf +/− allele at 255 basepair when comparing with a 100 basepair DNA ladder (Fermentas, Helsinborg, Sweden; Fig. 3).

Figure 3.
Gel electrophoresis showing Gdnf +/+, Gdnf +/− and Gdnf −/− genotypes. Tissue samples from Gdnf +/− animals showed two bands at 255 and 344 basepar while Gdnf +/± and Gdnf −/− animals showed just one allele at 344 and 255 basepair respectively. A 100 basepar DNA ladder was used to detect different gdnf allele.

Tissue preparation and immunohistochemistry

At designated time points, mice were anesthetized with an overdose of pentobarbital and cardioperfused with Ca^{2+}-free Tyrode solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4). In paper I, the heads from E19 fetuses of Gdnf +/+, Gdnf +/− and Gdnf −/− were collected and immersion fixed in 4% paraformaldehyde over night. The brains were harvested and kept in 10% sucrose in 0.1 M phosphate buffer containing 0.01% sodium azide in order to cryoprotect the tissue. Brains were frozen with gaseous carbon dioxide and then cryosectioned at 14 μm on a cryostat in papers I and II and at 12 μm, 14 μm, and 40 μm for paper III. The sections were collected on chromealun-gelatin-coated glass slides and proceeded for immunohistochemistry. The glass slides with the sections were washed in 0.1
M phosphate buffered saline (PBS) for 15 min. The primary antibodies were applied for 48 hours at 4°C (for information about the primary antibodies used in the studies see Table 1). All antibodies were diluted in PBS containing 0.3% Triton X-100. Washing steps 3 x 10 min with PBS (0.1 M) were performed before secondary antibodies were applied. After washing, goat serum (5% in 0.1 M PBS) was used to avoid unspecific binding of antibodies. The secondary antibodies, listed in Table 2, were applied for 1 h for 12 µm and 14 µm thick slices and for 4 h for 40 µm slices followed by washing in PBS (0.1 M) 3 x 10 min, and mounted in 90% glycerol in PBS to complete the immunohistochemical procedure.

**Stereology method**

Optical Fractionator design-based stereological method was used in paper III to estimate the total number of TH-positive neurons in the substantia nigra (Baquet et al., 2009; Gundersen et al., 1988; West et al., 1991). This method is independent of the size, shape, spatial orientation, and spatial distribution of the cells in the tissue. The substantia nigra was sectioned in a specific manner in order to collect sections for both cell counts and regular immunohistochemistry. Therefore 3 sections, 40 µm thick, were collected for stereology followed by 10 slices of 12 µm for immunohistochemistry and so on throughout the entire substantia nigra. Immuno-labelled cells for TH were counted in every 3rd 40 µm thick section using Olympus BX61 Microscope hard-coupled to MAC 5000 computer controlled x-y-z motorized stage (Ludl Electronic Products Ltd, Hawthorne NY), Optronics video camera system (MicroFire™ – Model S99808, Goleta, CA, USA) and StereoInvestigator 7 software (Document Version 4.00; MicroBrightField, Inc. Vermont, USA). A slice sampling fraction was thereby 1/6 since every 6th section was used with regard to the 10 sections of 12 µm used for immunohistochemistry.

Paxinos and Watson atlas was used to help to trace the substantia nigra and it was done under a low magnification (10X) on live microscopic video images. The first TH-positive neurons near the subthalamic nucleus were used as a rostral landmark. The caudal border of the substantia nigra was set at
the retrorubral field. The substantia nigra lateralis was included. Ventral tegmental area, which is located medially to the substantia nigra, was excluded by size and orientation of TH-positive neurons, which are smaller and not oriented along the long axis of the substantia nigra pars compacta. The medial lemniscus was used to delineate the substantia nigra posterio-medially (Nelson et al., 1996). Counting of TH-positive neurons was performed at high magnification (60X lens) with a systematic random design of dissector counting frames (100.5 x 75.67 µm), that was large enough to count about 3 TH-positive cells on average and at regular predetermined intervals (the grid area set to 180 µm x 180 µm). The height of counting frame was 20 µm and the actual mounted section thickness was found to be approximately 38 µm. The guard zone of 2 µm was used to avoid cutting artifacts at the upper and lower surfaces of the sections. Cells were counted in focus within counting area. The total number of neurons in the substantia nigra was estimated using the following equation:

\[
N = \frac{1}{ssf} \times \frac{1}{asf} \times \frac{1}{hsf} \times Q
\]

where \(ssf\) is the slice sampling fraction (\(ssf = 1/n = 1/6\)), \(asf\) is the area sampling fraction (\(asf = (100.5 \times 75.67)/(180 \times 180) = 0.24\), where (100.5 µm x 75.67 µm) is the dissector counting frame area and (180 µm x 180 µm) is the grid area), \(hsf\) is the height sampling fraction (\(hsf = 20/38\), where 20 µm is the height of the counting frame and 38 µm is the average thickness of the tissue), and \(Q\) is the number of counts from the counting frame. Estimated sampling error (CE) was calculated according to Gundersen formula which was modified to fit neuron counting by Keuker et al. and was within acceptable range and less than 0.1 (Gundersen et al., 1999; Keuker et al., 2001).
Table 1: Primary antibodies used in Papers I-III.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Target protein/Detection</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-rat TH</td>
<td>TH/Dopamine neurons</td>
<td>1:1500</td>
<td>Immunostar Inc.</td>
</tr>
<tr>
<td>Rabbit anti-human DARPP-32</td>
<td>DARPP-32/Striatal neurons</td>
<td>1:600</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-mouse GIRK2</td>
<td>GIRK2/A9 dopamine neurons</td>
<td>1:25</td>
<td>Millipore</td>
</tr>
<tr>
<td>Rabbit anti-mouse ALDH1A1</td>
<td>ALDH1A1/A9 dopamine neurons</td>
<td>1:100</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mouse anti-mouse Calbindin</td>
<td>Calbindin/A10 dopamine neurons</td>
<td>1:100</td>
<td>Sigma</td>
</tr>
<tr>
<td>Chicken anti-mouse Actin</td>
<td>Actin/cytoskeletal protein in all cells</td>
<td>1:10,000</td>
<td>Chemicon International</td>
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<tr>
<td>Rabbit anti-mouse α-synuclein</td>
<td>α-synuclein</td>
<td>IHC - 1:200, WB - 1:1000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-mouse phospho-α-synuclein</td>
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<td>Abcam</td>
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<tr>
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<tr>
<td>Rabbit anti-mouse Rab7</td>
<td>Rab7/endosomal protein</td>
<td>1:100</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>Mouse anti-mouse VDAC1/Porin</td>
<td>VDAC/outer membrane and plasma membrane protein</td>
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<td>Abcam</td>
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**Table 2: Secondary antibodies used in Papers I-III.**

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<th>Source</th>
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<td>Alexa Fluor®594 goat anti-mouse IgG</td>
<td>1:500</td>
<td>Invitrogen, Molecular probes™</td>
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<tr>
<td>Alexa Fluor®594 goat anti-rabbit IgG</td>
<td>1:500</td>
<td>Invitrogen, Molecular probes™</td>
</tr>
<tr>
<td>Alexa Fluor®488 goat anti-rabbit IgG</td>
<td>1:500</td>
<td>Invitrogen, Molecular probes™</td>
</tr>
<tr>
<td>Alexa Fluor®488 goat anti-mouse IgG</td>
<td>1:500</td>
<td>Invitrogen, Molecular probes™</td>
</tr>
<tr>
<td>Goat anti-rabbit immunoglobulins/HRP</td>
<td>1:10,000</td>
<td>DAKO, Cytomation</td>
</tr>
<tr>
<td>Immuno Pure IgG goat anti-mouse antibody</td>
<td>1:70,000</td>
<td>Pierce</td>
</tr>
</tbody>
</table>

**Western blot**

C57Bl/6j mice 6- and 22-month-old were anaesthetised with 4% isofluran (Baxter Medical AB, Sweden) and after neck dislocation the brains were rapidly dissected and placed in ice-cold saline. Cerebellum, striatum, and substantia nigra were dissected under a microscope and placed immediately in carbon dioxide ice for freezing. The tissues were stored at -75°C until the samples were prepared. The samples were thawed on ice and diluted with the lysis buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH = 7.2-7.4; 1% IGEPAL CA-630, 10% glycerol, 1% proteas inhibitor). Homogenization was performed using Sonifier B-12 (Branson sonic power company, Danbury, Connecticut) following by 15 min centrifugation at 4°C, 10,000 rpm. The supernatant and pellet were separated. The supernatant was used to determine α-synuclein and phospho-α-synuclein levels. The protein
concentration was determined by using the BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA). Eight µg protein from each supernatant samples were mixed 1:1 with loading buffer (5% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue, 0.125 M Tris HCl) and boiled 5 min at 95°C. Before the samples were loaded on 12% SDS polyacrylamide gels, they were cooled on ice. Precision Plus Protein™ Standards Dual Color (Bio-Rad) was used in order to determine the molecular weights of the proteins. The proteins were electro transferred onto nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Uppsala, Sweden) using the Trans-Blot Semidry transfer (Bio-Rad). Afterwards the membranes were blocked in 5% milk in Tris-buffered saline-tween (TBS-T) for 1 hour at room temperature in shaking conditions. Membranes were reacted with mouse anti-actin monoclonal antibody (1:10,000; Chemicon international), rabbit anti-mouse α-synuclein (1:1000; Cell Signaling), rabbit anti-human phospho-α-synuclein (PSP129; 1:10,000; Abcam) as primary antibodies and the secondary antibodies used were ImmunoPure IgG goat anti-mouse antibody (1:70,000, Pierce), polyclonal goat anti-rabbit immunoglobulins/HRP (1:10,000, Dako Cytomation, Denmark). Chemiluminescence signals were detected using ECL plus western blotting detection reagents and Hyperfilm ECL (GE Healthcare Life Sciences, Amersham Biosciences). Developer and fixer (1:5, Kodak, Sigma Aldrich) were used as processing chemicals for autoradiographic films according to manufacturers instructions. Band signal intensity was quantified by ImageJ 1.45s program.

**Evaluation and statistics**

All statistical evaluations were expressed as mean ± standard error of mean (SEM) and analyzed using SPSS 20 statistical software.

All transplants and E19 fetal brains in papers I and II were evaluated by fluorescence microscopy and estimation of the numbers of TH-positive neurons was performed in every fourth section through the entire transplants. TH-positive cells with visible cell nuclei were counted once since the TH-positive cells were estimated to be approximately 20 µm in diameter and the
section thickness was 14 µm. Due to the small volume of the transplant size, stereological method to calculate the cell number could not be applied in this case. Therefore, the estimated TH-positive cell number within the transplants cannot be accounted for the absolute cell number. Stereological method was used in paper III to estimate the number of TH-positive cells in the substantia nigra. Statistical evaluation was performed by one-factor analysis of variance (ANOVA) to detect differences between treatments or genotypes followed by Bonferroni post hoc test to determine pair wise differences. One-way ANOVA followed by Bonferroni as posthoc test was performed to analyze differences in behavior pattern in paper III. Two-factor ANOVA was performed to determine differences between groups over time (Papers I and III). ANOVA with repeated measures with the Greenhouse-Geisser correction was used in paper II to reveal differences in transplant size, measured by MRI, over time. Mann-Whitney test was performed to compare the size of VM and LGE transplants at different time points. $P < 0.05$ was considered statistically significant.
Results and discussion

The role of GDNF for maintenance of the nigrostriatal system

In papers I and II, transplantation of Gndf gene-deleted mice was used to investigate early as well as long-term effects of GDNF deficiency on VM dopamine neurons and striatal tissue organization and survival. Nigrostriatal microcircuits were created by co-grafting VM and LGE from E14 fetuses in different Gdnf genotype combinations. In addition, single transplants of VM and LGE were grafted into the lateral cerebral ventricles of 3-month-old wildtype mice (Fig. 2). The transplants were investigated at an early time point, 2-3 months postgrafting, and at later time points, up to 12 months.

Early effects of GDNF decrease or depletion

Survival of TH-positive neurons

A significant loss of TH-positive neurons were found at 2 months postgrafting in nigrostriatal double grafts with the genotype mismatch where Gdnf +/- VM was co-grafted with Gdnf +/- LGE (Fig. 2C and 4A). However, the survival of TH-positive neurons in VM co-grafts was not affected when LGE co-grafts were derived from tissue producing GDNF, even though when the VM portion of the co-grafts was derived from Gdnf +/- tissue (Fig. 4B). Interestingly, the co-grafts with both VM and LGE from tissue lacking GDNF survived 3 months postgrafting but not at the 6-month time point (Fig. 4C). It has been shown that GDNF mRNA is expressed in the developing but not adult brain in rodents (Stromberg et al., 1993). Later, using reverse transcription-polymerase chain reaction, the expression of GDNF mRNA was documented in the adult human brain, with the highest levels found in the striatum compared to other brain regions, while the levels were almost undetectable in the substantia nigra (Barroso-Chinea et al., 2005; Schaar et al., 1994). Still, a neuronal content of GDNF protein was found in the substantia nigra as well as neuronal expression of Ret and GFRα1, the receptors for GDNF (Chauhan et al., 2001; Golden et al., 1998; Trupp et al., 1996; Walker et al., 1998). This may indicate that GDNF is
retrogradely transported to dopaminergic neurons in the substantia nigra from the striatum (Mufson et al., 1999; Tomac et al., 1995b; Tsui and Pierchala, 2010). Thus, this mechanism may explain that the reduction of GDNF levels in the striatum seen from immature to mature tissue, results in decreased uptake, transport, and concentration of GDNF in the substantia nigra. Therefore, a possible downregulation of GDNF in the striatum may underlay the degeneration of the dopaminergic neurons in the substantia nigra seen in the co-transplants, where the striatum was derived from Gdnf+/− tissue.

**Figure 4.**
Cell counts of TH-positive neurons in VM-LGE co-grafts expressed as percentage of Gdnf+/− co-grafts in mismatch of Gdnf gene co-grafts with Gdnf+/− VM and LGE of all Gdnf genotypes (A), in double grafts with Gdnf+/− LGE and VM from different Gdnf genotypes (B), and in double grafts with the same Gdnf genotype for both VM and LGE (C). A significant loss of TH-positive neurons was seen in VM combined with LGE co-grafts derived from Gdnf−/− at 2 months (A). No difference in cell counts of TH-positive neurons was found in co-grafts when LGE was derived from Gdnf−/− tissue (B). No significant reduction in count of TH-positive neurons was found in double Gdnf−/− transplants at 3 months time point (C). *p < 0.05.

To identify A9 and A10 dopamine neurons in the VM portion of co-grafts, immunohistochemistry was used to identify neurons expressing GIRK2, ALDH1, and calbindin, respectively (Chung et al., 2005; Gerfen et al., 1985; Inanobe et al., 1999; Johansson et al., 1995; McCaffery and Drager, 1994; Meyer et al., 1999; Schein et al., 1998). The results revealed no difference in the number of subpopulations of dopamine neurons in grafts derived from different genotypes. Approximately 10% of total number of TH-positive neurons expressed GIRK2 and about 20% expressed calbindin (Fig. 2, Paper I). Thus, more neurons with an A10-like phenotype survived than A9 neurons. It is
known that the GDNF levels are higher in the ventral than the dorsal striatum (Barroso-Chinea et al., 2005). Both subtypes of TH-positive neurons require GDNF but at different doses, and degenerate in conditional GDNF knockout mice (Beck et al., 1995; Borgal et al., 2007; Kearns and Gash, 1995; Pascual et al., 2008; Tomac et al., 1995a). The results from Papers I and II partly confirms previous studies, i.e. the lack of GDNF led to degeneration of both subtypes of dopamine neurons in the VM portion of co-grafts at long-term but not at the short-term time points.

TH-negative inclusion-like structures were frequently found in transplants with great loss of TH-positive neurons thus, in transplants derived from Gdnf +/- and Gdnf +/− tissues already at early time points (Fig. 2, Paper I). Alpha-synuclein and ubiquitin immunohistochemistry was used in attempts to characterized these structures due to that these two proteins are major components of the Lewy bodies (Spillantini et al., 1997). Some inclusion-like structures were positive for α-synuclein and ubiquitin but some not, suggesting that these structures can not be characterized as Lewy bodies (Fig. 5, A and B).

**Figure 5.**
*Immunohistochemistry of TH-positive neurons for different endocellular markers were performed in attempts to investigate the inclusions-like structures. TH-immunoreactivity = green; α-synuclein (A), ubiquitin (B), VDAC (C), and Rab7 (D-F) = red. Some positive dots are matched to the inclusions-like structure (arrows) but some are not (arrowheads). Scale bar: 25 µm.*
Striatal tissue organization and survival

The size of single LGE grafts was significantly smaller than grafts derived from Gdnf /- tissue already at 2 weeks postgrafting as measured by MRI (Fig. 4, Paper II). Moreover, the size of LGE from Gdnf +/- tissue was significantly smaller in comparison to Gdnf +/- LGE from the 1-month time point and onwards (Fig. 4, Paper II). DARPP-32-positive neurons were located in large clusters in Gdnf +/- LGE, while only a few DARPP-32-positive neurons were present in very small grafts derived from Gdnf /- tissue at the later evaluation time point (Fig. 4, Paper II).

The TH-positive nerve fiber innervation of LGE portion of co-grafts from VM differed with the level of GDNF expression in the LGE tissue (Fig. 6), however, the GDNF expression in the VM had no influence on the TH-positive innervation. The pattern of innervation in LGE co-grafts with normal GDNF expression demonstrated TH-positive dense patches that overlapped with DARPP-32-positive regions (Fig. 3, Paper I and Fig. 6, Paper II). Already at 2 and 3 months postgrafting, reduction of GDNF production in LGE tissue (Gdnf +/-), altered the pattern of innervation to less dense TH-positive nerve fiber innervation of LGE with irregular overlap of DARPP-32-positive areas (Fig. 3, Paper I and Fig. 6, Paper II). Thus, the absence of GDNF in LGE co-grafts led to poor DARPP-32 expression and to a sparse and widespread TH-positive nerve fiber innervation of LGE (Fig. 3, Paper I and Fig. 6, Paper II). Interestingly, evaluation of the innervation and organization of the striatum in E19 Gdnf /- fetuses demonstrated that TH-positive neurons formed dense patches that overlapped with clusters of DARPP-32-positive neurons (Fig. 3, Paper I).

It has been reported that GDNF expression follows a patchy distribution that, in turn, potentially contribute to the same patchy dopaminergic innervation pattern of the developing striatum (Lopez-Martin et al., 1999; Olson and Seiger, 1972; Stromberg et al., 1993). On the other hand, lack or reduction of GDNF levels affect DARPP-32-positive neurons, which are the striatal output neurons and may possibly generate the non-patchy growth in striatal tissue of the transplants. Further, the dense patchy dopamine nerve fiber organization is observed only in the immature striatum, while the adult
striatum, which then has reduced GDNF mRNA levels, receives a more diffuse and widespread innervation (Stromberg and Johansson, 1994; Stromberg et al., 1997). Thus, the dopamine innervation pattern of immature versus mature striatum is similar to innervation pattern of LGE portion of co-grafts when LGE is derived from Gdnf-/+ and Gdnf-/- tissue, respectively. At birth, GDNF gene-deleted mice demonstrated no morphological impairment in the nigro-striatal system, which supports the normal appearance of the striatal organization in E19 Gdnf-/- fetuses (Fig. 3, Paper I) (Pichel et al., 1996). Thus, it seems that the organization of the striatum needs to pass the patchy stage, which involves high GDNF expression during development to become properly organized, dopamine innervated and functional later during adulthood.

**Figure 6.**
Schematic drawing of the innervation pattern of co-grafts with different genotype combinations. GDNF expression level in VM had no influence on the TH-positive innervation and DARPP-32-positive neuronal distribution in co-grafts. When LGE was derived from Gdnf-/+ tissue, TH-positive dense patches overlapped with DARPP-32-positive regions (A). In co-grafts with Gdnf-/- LGE, overlap of TH-positive nerve fibers with DARPP-32-positive areas was irregular (B). Gdnf-/- LGE influenced the TH-positive nerve fibers to form a sparse and widespread innervation and to a disorganization of DARPP-32 positive cells (C).
**Long-term effects of GDNF decrease or depletion**

*Survival of TH-positive neurons*

Counts of TH-positive neurons in single VM transplants revealed no significant differences between the genotypes at 12 months postgrafting (Fig. 5, Paper II). In addition, MRI demonstrated no significant difference in size of the transplants between the genotypes as monitored over one year (Fig. 4, Paper II). Thus, the results suggested that single LGE grafts are more vulnerable than VM to the reduction in GDNF levels.

In VM-LGE co-grafts of the same Gdnf genotype, significantly fewer TH-positive neurons were found in VM derived from Gdnf +/- tissue than in Gdnf +/- co-grafts at 6 and 12 months postgrafting (Fig. 1, Paper I). It seems therefore that GDNF is more important for the long-term maintenance of the dopamine neurons when they target the striatum than growing as single transplants, as seen in the view of TH-positive neuronal survival in the single VM grafts at 12 months. This might be explained by the fact that GDNF promotes presynaptic differentiation, formation of synapses, and synaptic efficacy of dopaminergic neurons (Bourque and Trudeau, 2000; Ledda et al., 2007). Neurons that never built synaptic contacts during development undergo apoptotic cell death, which can be prevented by GDNF (Janec and Burke, 1993; Oo et al., 2003). Thereby, the depletion of GDNF in LGE portion of co-grafts might lead to loss of TH-positive neurons probably due to poor striatal synapse formation, while neurons in the single transplants might still search for its target. Thus, TH-positive neurons co-grafted with LGE lacking GDNF seem to be more sensitive than neurons developed in VM without its striatal target.

*Striatal tissue organization and survival*

The significant decrease in size of single LGE transplants was reinforced with time and LGE transplants was undetectable at 6 months (Fig. 3 and 4, Paper II). One explanation might be the fact that GDNF mRNA levels is higher in the striatum during development than in the adulthood and consequently the striatum has greater requirement of GDNF to be maintained than VM. Thus, LGE is obviously more dependent on VM than VM is on LGE.
This argument is confirmed by the fact that LGE portion from Gdnf^{-/-} tissue in double grafts was not reduced to the same level as single LGE grafts.

The same effects of GDNF depletion on LGE organization were seen at later time points as seen in the earlier time points. Thus, the DARPP-32-positive neurons were not clustered and the TH-positive nerve fiber innervation was widespread (Fig. 4, Paper II).

**Blood capillaries and microglia in the co-grafts**

Glut-1-immunoreactivity, which visualizes blood capillaries, revealed that all transplants, independent of Gdnf genotype, had similar density and morphology. Further, Iba-1-immunoreactivity, a pan-microglia marker, demonstrated no difference in density of the microglia between transplants of different Gdnf genotypes over time. Interestingly, LGE had a slightly higher density of Iba-1-immunoreactivity compare to VM portion of co-grafts (Fig. 4, Paper I).

**Effects of small molecules on the nigrostriatal dopamine system**

The results from Gdnf deficiency in the survival of TH-positive neurons demonstrated inclusion-like structures in the dopamine neurons. These structures could not be characterized as Lewy bodies, however, α-synuclein can still play an important role in neuronal death. There is an idea that some endogenous protein can induce fibrillation of α-synuclein and thereby initiate Parkinson’s disease progression (Alim et al., 2002; Shtilerman et al., 2002). Therefore, small molecules that resemble dipeptides and can template or inhibit α-synuclein aggregation *in vitro*, named FN075 and MS382, respectively were used to further investigate the role of α-synuclein in degeneration of the dopamine neurons (Horvath et al., 2012; Horvath et al., 2013). The striatum was selected as the target due to the high levels of α-synuclein expression for injection of the small molecules (Fig. 2, Paper III).
Effects of injection into the striatum

Behavioral outcome

Behavioral tests that are sensitive to dysfunction in the nigrostriatal system were included in Paper III (Ogawa et al., 1987; Sedelis et al., 2001). To determine influences on spontaneous motor behavior, the cylinder test was used (Fleming et al., 2004; Fleming and Chesselet, 2006; Hwang et al., 2005). Motor coordination and balance was evaluated by the pole test (Fleming et al., 2004; Matsuura et al., 1997). Moreover, adhesive removal test was performed to test sensorimotor functions in mice (Fleming et al., 2004; Schallert et al., 1982). Animals receiving the templator performed significantly slower compared to control mice and mice injected with the inhibitor when adhesive removal test was performed. On the contrary, no significant differences in performance of the cylinder and the pole tests was found between mice given the templator compared to the vehicle (Fig. 1, Paper III). It is known that mice overexpressing human α-synuclein display early and progressive sensorimotor impairments (Fleming et al., 2004). Accumulation of α-synuclein in the brain, including the nigrostriatal system, but no loss of dopaminergic neurons was exhibited in these mice. In vitro studies have demonstrated that α-synuclein overexpression leads to dysfunction in dopamine synthesis and release (Lotharius et al., 2002; Lotharius and Brundin, 2002; Perez et al., 2002). Thus, behavioral impairments can be correlated to dopamine dysfunction without the loss of dopamine neurons.

Survival of TH-positive neurons in the substantia nigra after striatal injection

Cell counts of TH-positive neurons revealed no significant differences between animals injected with the templator, the inhibitor and the vehicle. However, some reduction in neuronal survival at 6 months after injection of FN075 was found (Fig. 2, Paper III). The number of TH-positive neurons was higher in animals that performed faster in behavioral adhesive test. The linear correlation coefficient (R) = 0.61; y = 84.77 - 0.0063 x (Fig. 1, Paper III).
Effects of injection into the substantia nigra

Survival of TH-positive neurons in the substantia nigra after nigral injection

At 3 months postinjection, a significant loss of TH-positive neurons was found in the substantia nigra of animals that had received FN075 compared to controls (Fig. 3, Paper III). TH-negative inclusion-like structures, similar to those found in the transplants, were seen in the remaining dopamine neurons after injection of the templator both into the striatum and into the substantia nigra. To further investigate the inclusion-like structures, immunohistochemistry to visualize the mitochondria was performed due to some investigators' statement that mutations or the inhibiton of the mitochondrial complex I can be a possible underlying mechanism in Parkinson's disease pathogenesis (Di Monte et al., 1992; Langston et al., 1983; Parker et al., 1989; Schapira et al., 1989). The mitochondrial outer membrane and the plasma membrane marker, VDAC, was initially used to detect the mitochondria in dopamine neurons with inclusions-like structures. Some inclusions-like structures were matched with VDAC-positive dots (Fig. 5, C). Interestingly, the mitochondria marker TOM20, which reacts with the outer membrane, demonstrated enlarged or aggregated mitochondria in dopamine neurons with the inclusion-like structures (Fig. 7, D-F). Mice overexpressing α-synuclein have shown similar mitochondrial dysfunction, indicating an early phase in the process of cell destruction (Choubey et al., 2011; Sterky et al., 2011). It was demonstrated that both monomeric and aggregated α-synuclein can be secreted by exocytic and endocytic pathways (Danzer et al., 2011; Lee et al., 2005). Additionally, α-synuclein is present in endosomal compartments and α-synuclein secretion and lysosomal targeting is regulated by vacuolar protein sorting 4 (Hasegawa et al., 2011). Therefore, the inclusion-like structures were tested for the endosome marker, Rab7, which is located in late endosomes and control membrane transport from early to late endosome and to lysosomes (Feng et al., 1995; Meresse et al., 1995). Some inclusion-like structures were positive for Rab7, but others not, suggesting that not all these structures can be addressed to endosomes (Fig. 5, D-F). Thus, it was not possible to fully characterize the inclusion-like structures, although vague changes in
mitochondria and endosomes were indicated. However, it was clear that these cytoplasmic inclusions were frequently present in A9 TH-positive neurons of animals affected with loss of dopamine neurons, while A10 neurons were spared.

Figure 7.
TOM20-immunohistochemistry of TH-positive neurons demonstrated that TH-positive cells without inclusions-like structures have even distribution of mitochondria markers in cytoplasm (A-C). In contrast, when inclusions-like structures appeared, the mitochondria were enlarged or aggregated (D-F). TH-immunohistochemistry = green, TOM20-immunohistochemistry = red. Scale bar 12.5 μm.
Concluding remarks

- GDNF is important for long-term maintenance of both VM and LGE and for dopamine axonal guidance and proper organization of the striatum.
- Brain levels of α-synuclein are high in the striatum.
- Striatal injection of FN075 causes behavioral impairment in mice without affecting the number of TH-positive neurons in the substantia nigra at 6 months.
- Substantia nigra injection of FN075 affects the nigrostriatal system by significant loss of TH-positive neurons at 3 months.
- TH-negative inclusions-like structures were found in the transplants lacking GDNF as well as in dopamine neurons after injection with FN075 into the striatum and the substantia nigra.
- Mice injected with small molecules that modulate endogenous α-synuclein may become a useful Parkinson's disease model.
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