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NTS-polyplex: A potential nanocarrier for neurotrophic therapy of Parkinson's disease

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Abstract

Nanomedicine has focused on targeted neurotrophic gene delivery to the brain as a strategy to stop and reverse neurodegeneration in Parkinson's disease. Because of improved transfection ability, synthetic nanocarriers have become candidates for neurotrophic therapy. Neurotensin (NTS)-polyplex is a "Trojan horse" synthetic nanocarrier system that enters dopaminergic neurons through NTS receptor internalization to deliver a genetic cargo. The success of preclinical studies with different neurotrophic genes supports the possibility of using NTS-polyplex in nanomedicine. In this review, we describe the mechanism of NTS-polyplex transfection. We discuss the concept that an effective neurotrophic therapy requires a simultaneous effect on the axon terminals and

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soma of the remaining dopaminergic neurons. We also discuss the future of this strategy for the treatment of Parkinson's disease.

Keywords

Neurorestoration; neuroprotection; neurodegeneration; regeneration; survival

Parkinson's Disease (PD) results from a progressive loss of dopaminergic neurons in the substantia nigra with concurrent gliosis in the midbrain.[1] These early-stage events in PD result in dopamine depletion in the caudoputamen, with the lateral nigral projections to the putamen being the most affected.[2] As the disease advances, positron emission tomography shows a progressive loss of dopamine storage in striatal, cingulate and frontal brain regions.[3]

The earliest and most striking physical disabilities resulting from these changes in the basal ganglia are motor impairments. These include paucity and slowness of movement (akinesia, bradykinesia), muscle stiffness (rigidity), tremor at rest, and postural instability. An increasing number of clinical reports suggest that several nonmotor symptoms of PD, such as sleep disorders, autonomic dysfunction, neuroendocrinal problems, and neuropsychiatric symptoms (anxiety, apathy, dementia, cognitive dysfunction, and depression) also result in part from dopaminergic impairment.[4] These nonmotor symptoms of PD are thought to be caused by reduced dopamine neurotransmission in more than one area of the cerebral cortex, diencephalon, and hypothalamus (Figure 1). In the rat, individual dopaminergic neurons of the substantia nigra profusely branch out to innervate several brain structures, including the striatum, the globus pallidus, the subthalamic nucleus, the substantia nigra reticulata, the reticular thalamic nucleus and the cerebral cortex.[5–12] Such extensive axonal divergence may explain in part why loss of dopamine neurons results in reduced dopaminergic transmission in multiple structures and generates the multiple motor and nonmotor symptoms of PD (Figure 1). Consequently, an effective therapy for PD should aim to stop dopamine neuron degeneration and repair not only the nigrostriatal pathway but also the projections of nigral dopaminergic neurons in multiple target areas.

The progressive character of dopaminergic neurodegeneration and the availability of new methods allowing early diagnosis have encouraged the use of neurotrophic therapy for PD.[13,14] The residual dopaminergic neurons and terminals are a significant substrate on which neurotrophic factors can act to promote cell survival and axonal repair. Differently from the use of neurotrophic proteins, which require repeated injections or continuous infusion using pumps, gene therapy has the advantage of providing sustained production of the protein following a single administration of its gene.[15] Thus, gene therapy for PD aims to insert a foreign neurotrophic gene (transgene) within the cells of a brain region, where the released transgenic protein can simultaneously reach the somatodendritic and terminal compartments of nigral dopaminergic neurons to slow down their progressive cell death and restore their functional connectivity with target nuclei.

Today, a majority of gene therapy protocols for PD use viral vectors to insert neurotrophic transgenes into cells of either the striatum or the substantia nigra.[16] These two approaches have yielded different results in experimental animals depending on the time of injection, the neurotrophic factor, and the viral vector used. In general, transduction of striatal cells leads to sprouting and regrowth into the area of transgenic neurotrophic factor expression, along with a varying level of functional recovery. In contrast, injection of the vector into the substantia nigra protects the nigral cell bodies and causes extensive local sprouting of tyrosine hydroxylase (TH)-positive fibers, but they are unable to functionally reinnervate the striatum.[17] To date, only one gene-therapy approach for PD has been tested in the putamen

of human patients and involved the use of neurturin (NRTN), a member of the glial cell-line-derived neurotrophic-factor (GDNF) family ligands.[18] Notable time-dependent improvements in a variety of clinical ratings were reported in patients in a phase I trial of adeno-associated virus (AAV)-based NRTN-gene therapy.[19] Data from a Phase II trial in 58 patients with advanced PD showed a clinically modest benefit after 18 months of treatment [Press release on 27 May 2009 by Ceregene Inc.]. The trial's sponsor also reported clear evidence of NRTN expression in the targeted putamen but no evidence for transport of this protein to the cell bodies of the degenerating dopaminergic neurons. Scarcity of the nigrostriatal terminals surviving in advanced PD might limit the retrograde transport of the AAV vector and-or NRTN to the nigral cell bodies. This is a limiting factor for all forms of neurotrophic gene therapy for PD, especially considering the extensive loss of divergent innervation of multiple structures following degeneration of dopaminergic neurons.

NTS-polyplex is the first nonviral system for gene delivery with a proven ability to transfect dopamine neurons of the substantia nigra in vitro and in vivo (Figure 2).[20–23] Previous studies in the 6-hydroxydopamine (6-OHDA) rat model show that the NTS-polyplex transfection of a neurotrophic gene into the substantia nigra of rats after the neurotoxin injection produces biochemical, anatomical, and behavioral recovery from Parkinsonism.[24–26] These findings have raised the interest of using NTS-polyplex in the neurotrophic therapy for Parkinson's disease (PD), thus making it important to analyze its transfection mechanism and its possible usefulness in neurotrophic therapy for PD. In this review, we propose the hypothesis that the NTSR1-mediated endocytosis is the major route of the NTS-polyplex internalization and of the consequent transgene expression in vitro and in vivo (Figure 3).[20,22,27] Because of its mechanism of entry and control of transgene expression by a promoter derived from a dopamine cell-specific gene, the NTS-polyplex has become an efficient, cell-specific, and sustained strategy for transgene expression in dopaminergic neurons. Based on this feature, we also propose that the NTS-polyplex transfection of a neurotrophic gene into the residual dopaminergic neurons of PD patients will be able to provide a simultaneous effect on the cell bodies and terminals of those neurons. The expressed neurotrophic protein will be released by cell bodies and, after its anterograde axonal transport, by axon terminals. Finally, we discuss the limitations, perspectives, and possibilities of the NTS-polyplex nanoparticle system in a neurotrophic therapy for PD and other neurodegenerative diseases of the central nervous system.

NTS-polyplex-mediated gene transfer

Gene-therapy protocols for PD have not paid much attention to nonviral vectors for neurotrophic gene delivery. One of the reasons may be that many consider such vectors as not being able to provide sustained gene expression in the brain. [28] NTS-polyplex was the first nonviral system that showed long-lasting transgene expression in dopaminergic neurons of the substantia nigra in the rat brain.[20–22] NTS-polyplex consists of nanoparticles (Figure 4, D) resulting from the electrostatic binding of the conjugate NTS–poly-L-lysine (NTS carrier) to plasmid DNA (pDNA). The NTS carrier takes advantage of the endocytosis of NTS with its high-affinity receptor (NTSR1) to transfer the pDNA into dopamine neurons.[20,21] In addition, the NTS-polyplex incorporates a fusogenic peptide (FP) and a karyophilic peptide (KP) of viral origin that enhance transfection efficiency (Figure 5). The FP is also cross-linked with the poly-lysine moiety; its role is to rescue NTS-polyplex from endosomes before the enzymatic degradation and extreme acidity become prevalent[22,29] (Figure 5). The KP is a nuclear localization signal that is electrostatically bound to the pDNA to promote its import to the cell nucleus.[22,30]

Plasmid DNA condensation

Condensation of pDNA into a small, compact structure, is the first decisive step for gene transfer mediated by cationic polymers.[31] This process is directed by electrical forces resulting in spontaneous formation of polyplexes when an equimolar ratio between the cationic polymer and the plasmid DNA is achieved.[32] The resulting particles are toroid structures or rods with a size of 50 to 100 nm.

Several cationic polymers have been used to compact both ribonucleotides and deoxyribonucleotides (polyanionic) because of their electrostatic bonding properties. The reacting group of polymers includes primary, secondary, tertiary, and quaternary amines or other positively charged molecules such as amidines.[33] To date, the cationic polymers mostly used in gene delivery are spermidine, protamine, polyhistidine, polyarginine, and polylysine.[34] Poly-L-lysine is an attractive molecule to use in human gene-therapy because it has been tested as a coadjuvant for prolonged release of certain drugs with proven clinical efficacy and good biosafety results. Its clinical use relies on its exceptional solubility, absence of acute toxicity, and inability to cause immunogenicity with repeated doses.[35] The epsilon-amino groups of poly-L-lysine are positively charged at physiological pH, which represents a particularly advantageous chemical property. This charge allows poly-l-lysine to bind the negatively charged pDNA to form the polyplex under physiological conditions. In addition, the epsilon-amino groups are good acceptors of molecules active in receptor-meditated endocytosis, such as proteins, peptides and sugars, which are incorporated into poly-L-lysine by using chemical crosslinkers, nonenzymatic glycosylation, or avidin-biotin technique.[34,36]

A polyplex nanoparticle based on polylysine comprises various pDNA molecules covered by quite a few polymeric chains, whose structure, size, and solubility depend on the following factors: 1) the ratio of electrostatic charges of pDNA and poly-L-lysine, 2) the size and ramification grade of poly-L-lysine, 3) the physical and chemical conditions used for polyplex formation, and 4) the sequence used to mix the reactants during polyplex formation.[21] The pDNAconcentration is also a factor influencing polyplex solubility; concentrations less than 20 μg/mL are required to form a soluble complex. Interestingly, the polyplex nanoparticle size is independent of the pDNA size.[37]

An electron-microscopy study showed that the electrostatic binding of the NTS-poly-L-lysine conjugate (the NTS-carrier) to a pDNA produces mostly toroidal nanoparticles of NTS-polyplex with an average diameter of 100 nm (Figure 4, D).[21] This study also showed that both the KP (6 μM) and fetal bovine serum (1%) caused partial condensation of pDNA (6 nM), which is further compacted to a toroid structure by the addition of the NTS-carrier (Figure 4, A–D).[21] This precondensation may be caused by the interaction between the negatively charged pDNA, the positively charged KP, and the cationic proteins of the serum. The addition of serum is known to stabilize the structure of the neutral polyplex at physiological salt concentrations, thus avoiding the rapid formation of large aggregates,[38] which are generally ineffective gene-delivery agents and can even be dangerous because of embolization of the particles in the lung.[39] In the process of NTS-polyplex formation, serum is more effective if added before the NTS-vector.[21] The subsequent addition of the NTS-carrier produces neutral nanoparticles (Martinez-Fong et al., unpublished results) that are unable to react with the electrically charged serum protein, thus avoiding the formation of large aggregates and embolization when injected intravenously. The absence of circulatory problems when NTS-polyplex is repeatedly administered through the blood stream was recently shown in an animal model of cancer gene-therapy.[40]

In summary, the NTS-polyplex nanoparticles must fulfill two conditions to cause efficient transfection; an adequate condensation of pDNA into a toroid structure and a sufficient

concentration of these structures at an optimum molar ratio.[21] Because the cellular entry of nanomaterials depends on electrical charge, particle size, and particle shape [41], further characterization of the NTS-polyplex nanoparticles should be accomplished using different techniques, such as Rx-analysis, atomic force microscopy, scanning electron microscopy, and dynamic light scattering to determine size and z-potential. The knowledge of these characteristics will be useful in the formulation of the NTS-polyplex for clinical application in PD.

Receptor-mediated endocytosis

In mammalian cells, clathrin-coated pits and vesicles provide a well-defined pathway for internalizing extracellular material, ligands and plasma membrane. In this process, called receptor-mediated endocytosis, macromolecules bind to complementary, transmembrane receptor proteins, accumulate in coated pits and then enter the cell as receptor-macromolecule complexes in clathrin-coated vesicles.[42] The tridecapeptide NTS in the NTS-polyplex is the natural ligand of three receptors, NTSR1, NTSR2, and NTSR3, which internalize NTS upon ligand activation in cells of the human and rodent brain.[43,44] Of these three NTS-receptors, results from internalization and expression assays showed that NTSR1-mediated endocytosis is the major route of NTS-polyplex internalization and of the consequent transgene expression *in vitro* (Figures 6 and 7) and *in vivo*.[20,27,45] N1E-115 and human colonic-adenocarcinoma HT-29 cells were selected as a gene-therapy model *in vitro* because they express NTSR1[46] but not NTSR2.[47] They were also used to validate the ability of NTSR antagonists or endocytosis blockers to prevent fluorescent-NTS-polyplex uptake and reporter-gene expression.[22,27] This strategy, validated *in vitro*, was used to demonstrate the specificity of transfection *in vivo*.[20,22] In the brain, the highest density of NTSR1 RNA and binding sites have been found in the ventral mesencephalon, followed by hypothalamus, prefrontal cortex, striatum, and cerebellum.[47–51] Of the two mesencephalic nuclei rich in NTSR1, the substantia nigra was selected for NTS-polyplex transfection because its dopaminergic population possesses the highest density of NTSR1 binding sites in the brain.[52,53] The internalization and expression studies yielded similar results *in vitro* and *in vivo*, thus supporting the participation of NTSR1 in NTS-polyplex transfection.

In internalization studies, either calcein or TH immunofluorescence were used when using confocal microscopy to delimit the cell area and locate the NTS-polyplex harboring a propidium iodide-labeled plasmid DNA within the cell. Confocal microscopy showed the fluorescence of propidium-iodide within calcein-stained cells *in vitro* (Figure 3)[22,27,45] and in TH-stained neurons [20,22] thus suggesting the intracellular presence of NTS-polyplex. Interestingly, the kinetics of NTSR1 to internalize the NTS-polyplex were similar to that of NTS or NTS agonist internalization in cultured neurons from the brain of mouse and rat embryos and cell lines.[54] In those cells, the radioactive ligand and the GFP- or epitope-tagged receptor were rapidly removed from the cell surface and clearly located within cytoplasmic vesicles during the first 15 to 30 min.[54–56] In TH-positive nigral neurons, nuclear propidium iodine signal was detected in the cell 4 h after local injection of NTS-polyplex.[20] The blockade of pDNA uptake by either an excess of NTS or the NTSR1 antagonist SR-48692[57] confirmed both *in vitro* and *in vivo* that NTS-polyplex resulted from NTSR1 internalization.[20,22,27] The absence of similar uptake under conditions where clathrin-coated pit formation was blocked by hypertonic sucrose[58] also provided direct support for the idea that receptor-mediated endocytosis is the mechanism used by the NTS-polyplex to internalize in cells.[20,22,27] Accordingly, the transfection of reporter genes (green fluorescent protein and chloramphenicol acetyl transferase) using the NTS-polyplex led to transgene expression only in NTSR1-bearing cell lines and nigral dopaminergic neurons. Expression was also absent in cell lines lacking NTSR1, such as COS-7 and L-929 cells.[20,22,27]

Similar to NTSR1, NTSR2 is also a G-protein-coupled receptor [59,60] which internalizes after activation by agonists.[61] However, no transgene expression was seen when NTS-polyplex was injected into the ansiform lobule of the cerebellum, a region rich in NTSR2.[48,62] In addition, astrocytes of the substantia nigra, known to express NTSR2,[49] were unable to internalize the NTS-polyplex and express reporter genes.[20,22] Internalization and expression assays in primary cultures of substantia nigra glial cells confirmed that glial NTSR2 does not mediate NTS-polyplex transfection.[20] These cells show only membrane binding of the NTS-polyplex, which is blocked by 1 μM levocabastine, a competitive antagonist of NTSR2.[47,59]

NTSR3 is a single transmembrane-domain receptor, which is 100% homologous to gp95-sortilin,[63] mainly localized in the trans Golgi-network, and poorly expressed at the plasma membrane.[64] Because NTSR1 and NTSR3 are able to form a complex to internalize NTS in HT29 cells,[65] the participation of the NTSR3 in NTS-polyplex endocytosis cannot be ruled out. It would be worthwhile to explore this issue when selective pharmacological ligands for NTSR3 become available.

Quantitative studies on the NTS-polyplex components determined that a functional NTS-polyplex provides sufficient NTS to activate NTSR1-mediated endocytosis *in vitro* and *in vivo*. Using [^{125}I]NTS, it was determined that an efficient NTS carrier contains one molecule of NTS and two molecules of poly-L-lysine.[21,22] Based on the equimolar ratio between pDNA and the NTS carrier, those authors determined that the concentration of NTS provided by a functional NTS-polyplex depends on the plasmid.[21] For instance, the optimal NTS concentrations were 253 nM for pEGFP-N1 and 745 nM for pDAT-EGFP.[21] These NTS concentrations are at least 50 times higher than the affinity constant (Kd) of NTS binding to NTSR1 in N1E-115 cells (5 nM)[66] and dopamine neurons (0.3 nM),[67] thus assuring maximal activation of NTSR1-mediated endocytosis.[55]

NTSR1-mediated retrograde transport of NTS from axonal terminals to nigral cell bodies could be another route for the NTS-polyplex transfection of dopaminergic neurons that can be useful in experimental studies and future clinical trials. This retrograde transport has been extensively documented in the nigrostriatal and mesolimbic pathways and it is possible that it occurs in the collaterals innervating the other targets of dopaminergic neurons.[68] Taking advantage of this route of NTS transport toward nigral dopaminergic neurons, it is feasible to transfect nigral neurons after injection of NTS-polyplex into the striatum.[21] The involvement of NTSR1 in the uptake of NTS-polyplex and in subsequent expression of the reporter gene was confirmed by the absence of transgene expression in experiments using either NTS-polyplex in the presence of the selective NTS-receptor antagonist SR-48692[57] or an untargeted polyplex (this contains all the molecular components of NTS-polyplex except NTS).[21]

Endosomal escape

Shuttling through the endosome is a natural port of access to the cell cytoplasm. Cells use endosomes to incorporate extracellular material needed for normal physiological functions, signal transduction and cell signaling through phagocytosis and regulated endocytosis.[69] Endocytosis can be triggered by activating cell-surface receptors that are clustered at plasma membrane invaginations and further enclosed into membrane vesicles.[42] This mechanism has been used by viruses that activate cell-surface receptors leading to the formation of endosomes.[70] In an effort to fight these noxious intruders, cells have created a natural barrier to respond to the threat of exogenous bioparticles. The response is a natural defense that uses acidification of the endosome and pH-activated proteases, lipases and nucleases to denature incoming particles.[71] Itis well known that acidification triggers denaturation of proteins and nucleic acids by changing their secondary and tertiary structure. However,

several viruses have evolved strategies to overcome this cellular defense mechanism: for example, some pH-sensitive viral envelope proteins change their tertiary structure when the pH reaches a certain acidic point inside the endosome. [72] The proteins thus adopt a new tertiary structure that is rich in α-helix, imparting a lipophilic property that disrupts the endoplasmic membrane.[73,74] The result of this viral peptide-mediated endoplasmic membrane fusion is the escape of the virus to the cell cytoplasm.[72]

Similar to viruses, the first limiting factor of nonviral vectors is their inactivation in acidic endosomal vesicles[75] and their degradation in the lysosomal compartment.[71,76] To overcome the first barrier, diverse approaches have been successfully used together with receptor-mediated gene-transfer systems, including hepatectomy-induced liver regeneration after the injection of a asialoglycoprotein-polyplex,[77] replication-defective adenovirus to cause disruption of the DNA-containing endosomes,[78] histidylation of polylysine to destabilize the endosome membrane,[79] and chloroquine to neutralize the acidic pH of the endosomes.[80]

A 22 amino acids peptide (GLFEAIAEFIEGGWEGLIEGCA) from the amino-terminus of the influenza virus hemagglutinin HA2, capable of fusing with the endocytotic vesicle lipid bilayer has been isolated and characterized.[29,81] The addition of this FP to the culture media of hepatoma cells significantly increased gene expression when the cells were transfected via receptor-mediated endocytosis.[81] Based on this report, a synthetic replica of this FP was cross-linked to poly-L-lysine of NTS-carrier to provide more effective, targeted gene delivery *in vivo*(Figure 5).[22] This conjugate has considerable benefits because it combines activation of NTSR1-mediated endocytosis and a mechanism for escape from endosomes. Quantitative studies on NTS-polyplex components using [^{125}I]NTS and [^{3}H]-FP determined that a molecule of NTS-carrier contains one molecule of NTS, four molecules of FP, and two molecules of poly-L-lysine.[21] Quantitative techniques demonstrated that the presence of the FP in the NTS-vector improved both pDNA internalization and the subsequent expression of the reporter gene *in vitro* and *in vivo* by more than 300%.[22] This improvement might have resulted from an increased amount of exogenous DNA in the cytoplasm after endosomal membrane disruption by the FP (Figure 7). Remarkably, the NTS-polyplex keeps its specificity despite the addition of FP, as demonstrated by the absence of gene transfer in NTSR1-lacking COS7 cells and in N1E-115 cells incubated with SR-48692 to block NTSR1-mediated endocytosis. These results further confirm that the FP is inactive at neutral pH such as that of the extracellular medium (Figures 6 and 7).[22]

Dissociation or precipitation of the NTS-polyplex might occur because of the acidic pH to which it is exposed during its passage through the endosome prior to reaching the nucleus. However, electrophoresis analysis in a pH gradient showed that the presence of the FP in the NTS-polyplex contributes positively to its integrity and stability at pH 6.0.[21] Mechanistic studies have shown that, at neutral pH, FP exists in a non-fusogenic state, but upon exposure to low pH, an alpha-helix conformation of the structure occurs to expose a fusogenic activity.[74] It is possible that this mechanism is conserved in the FP of NTS-polyplex and that this peptide changes conformation at acidic pH and destabilizes the endosomal membranes thus resulting in an increased cytoplasmic gene delivery.

In summary, the results analyzed above clearly establish that the incorporation of the FP into the NTS-polyplex is an efficient strategy to improve the efficiency of gene transfer *in vivo*.[21,22] However, the route of the NTS-polyplex through the intracellular compartments is not completely characterized. In addition, other techniques should be used to confirm and give details about the intracellular trafficking; for details see reference # 41. After endosomal escape of the NTS-polyplex, the precious DNA “cargo” must traverse the

cytoplasm to reach the nucleus where it will be transcribed into mRNA of a therapeutic peptide.

Nuclear targeting

The nuclear membrane is endowed with receptors that function as check points to limit access to the nucleus. To pass through the nuclear membrane and activate nuclear receptors, proteins or RNA need appropriate conformations or “access codes”, a sophisticated security mechanism that viruses have evolved to bypass. The mechanisms responsible for nuclear import of viral cargos and the insertion of viral DNA in the host genome can be exploited by gene-therapy approaches. However, the insertion of viral DNA can activate proto-oncogenes or cause mutations, which might result in cancer. To improve transfection efficiency without activating proto-oncogenes, nonviral vectors must be designed to take advantage of nuclear-import but not viral DNA insertion mechanisms.[82]

Once the virus reaches the cytoplasm of the infected cell, some viral proteins possessing karyophilic determinants, also known as the nuclear-localization signal (NLS), import the virus genome and functional viral proteins to the nucleus of the host cell.[83] In 1984, Kalderon and coworkers characterized a nuclear-targeting signal in the simian virus 40 (SV40) large-T antigen and described the first nuclear-import system.[84] Since then, several NLS and pathways for nuclear transport have been described, of which the classical nuclear-import pathway is the best characterized.[83] The NLS of the SV40 major capsid-protein Vp1 is responsible for nuclear targeting of Vp1 and virions.[85] Further analysis of Vp1 NLS has shown that a mutant19-amino acids long (MAPTKRKGSCPGAAPNKPK) peptide has potent nuclear-import activity.[30] To increase transfection, this viral peptide was integrated in NTS-polyplex. This strategy intends to introduce exogenous DNA into the cell nucleus and force cells to transcribe, translate and put to work transgene products without affecting normal cell function.

The SV40 Vp1 NLS (KP) was selected because of its potent import activity and the simplicity of incorporating it into the NTS-polyplex.[22] The presence of four lysines in the peptidic backbone of KP allows it to bind electrostatically to plasmid DNA before the addition of the NTS-vector to form the NTS-polyplex[22] (Figure 4, B). Evidence of the incorporation of KP into the pDNA and of pDNA condensation was provided by transmission electron microscopy and agarose-gel electrophoresis.[21] The electrostatic interaction between the KP and pDNA caused retardation in the mobility of the pDNA in an agarose-gel electrophoresis confirming the formation of a KP-pDNA complex.[21]

The incorporation of viral peptide in the NTS-polyplex produced an efficient and durable expression of transgenes in cultured cells and *in vivo* in the rat (Figure 2). Quantitative analysis showed that the presence of only the KP in the NTS-polyplex (lacking the FP) increases transgene expression *in vitro* by approximately 50%, which increases to > 600% when the NTS-polyplex contains both the FP and KP (Figures 6 and 7).[22] These data strongly suggest that the KP remains bound to pDNA after the addition of the NTS-vector, even though it contains polylysine. This suggestion is further supported by the finding that a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus.[86] In addition, there is evidence in the gene therapy literature that NLS mediated gene transfer still works after irreversible chemical linkage of the NLS-peptide to cDNA. [87,88]

Similarly, the combined use of the endosomal neutralizing agent chloroquine and of the SV40 large-tumor-antigen NLS improves the transfection efficiency of transferrin-polyplex.[80,89] The successful use of strategies to escape from the endosome or to inhibit endosomal acidity strongly supports the idea that the major barrier to receptor-mediated gene-transfer systems is acidification of endosomal vesicles.[90] Because of its capacity for

cell entry, endosomal escape and nuclear import, the NTS-polyplex thus has the ability to provide efficient and durable transgene expression without the requirement for complementary treatments such as chloroquine, which increases the transfection efficiency by preventing endosome acidification.[91]

The use of a synthetic peptide in the NTS-polyplex has considerable benefits over the use of mutated or chemically modified viruses. It results insmaller complexes and considering the lack of toxicity or of any potential infectivity, the technique holds great promise for therapeutic intervention, especially for PD. The most striking benefit of merging the viral strategy with the transfection specificity of NTS-polyplex is the ability to achieve durable transgene expression in dopaminergic neurons of the substantia nigra. The injection of the NTS-polyplex into the substantia nigra of rats produces strong transgene expression in large numbers of dopaminergic neurons but not in nearby glial cells (Figures 2 and 9).[21–24] The blockade of NTSR1 binding site by NTS-antagonists and the ineffectiveness of an untargeted polyplex (lacking NTS) clearly demonstrated that NTSR1-mediated endocytosis plays a key role in gene transfer *in vivo*.[21,22] Transgene expression has been detected for up to two months (the end of the study) after NTS-polyplex injection and the observation of high levels of transgene expression at the study end point suggests that it can endure even longer.[22–24]

In summary, although the involvement of the NTSR1 receptor in the NTS-polyplex transfection is solidly demonstrated in vitro and in vivo, the route of the NTS-polyplex through the intracellular compartments and the import mechanism into the cell nucleus remain to be clarified by using different techniques; for details see reference # 41. In addition, the identification of the nuclear compartment where the transgene is lodged and the molecular mechanisms of transgene transcription and regulation are still unknown.

Tissue-specific promoters

A high degree of dopamine cell-specificity of transgene expression has been achieved with the NTS-polyplex approach, based on the distribution of NTSR1integral to the transfection process and the ability to target NTS-polyplex injections into the immediate vicinity of dopaminergic neurons or their terminals (see above). It might be possible to further enhance cell-specificity of expression by placing the transgene under the control of a promoter derived from a dopamine cell-specific gene (Figure 5). The dopamine transporter (DAT) is a plasma membrane protein that functions to clear released dopamine from the extracellular space, thereby controlling the amplitude and duration of dopamine signaling.[92] The DAT gene is most robustly expressed within substantia nigra dopamine neurons (those cells most impacted in PD), with a specificity of cell expression unrivaled by any other genes associated with the dopaminergic phenotype.[93] Recently, an NTS-polyplex containing the BDNF-flag gene under control of the previously characterized[94] human DAT promoter sequence, was injected into the striatum, a tissue known to contain high levels of NTSR1.[48] As expected, the human DAT promoter prevented transgene expression in these striatal cells.[21] In contrast, BDNF-flag expression was detected in dopaminergic neurons of the substantia nigra due to NTSR1-mediated retrograde transport of the transgene from striatal terminals. The retrograde transport of NTS by NTSR1 is well-characterized in experimental animals[68] and represents an alternative route of transfection of nigral dopaminergic neurons. In addition, the use of tissue-specific promoters might prolong NTS-polyplex-mediated gene expression because DAT promoter activity is not expected to be transcriptionally silenced by methylation, as is the case for viral promoters such as CMV.[95] In the future, it might be possible to modify the DNA sequence, or pharmacologically manipulate the functional activity of tissue-specific promoters to provide even more refined control of transgene expression.

Neurotrophic therapy

Traditionally, the term neurotrophic factors (NTFs) has referred to a group of secreted proteins that regulate the life and death of specific sets of neuronal subpopulations during development. The role of NTFs is well-established in CNS development. In contrast, the function of NTFs in the survival, maintenance and plasticity of the mature CNS is less clear. Nevertheless, evidence in the adult brain showing that NTFs promote neuronal regeneration following diverse types of injury has encouraged their use in the treatment of neurodegenerative disease and neural trauma.

The recent discovery and characterization of two new evolutionarily-conserved NTFs, cerebral dopamine-neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF), has augmented the families of NTFs to four groups.[96,97] The modern classification[98] includes:

1. the neurotrophins: Nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and NT-4[99]
2. GDNF family ligands (GFLs): GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN)[18,100]
3. neuropoietic cytokines (also referred as the interleukin-6 (IL-6) family)[101] and
4. the novel CDNF-MANF family.[96,102]

NTFs act on neurons through transmembrane receptors with intrinsic tyrosine kinase activity or via other receptor-associated kinases.[100] The receptors for CDNF and MANF are presently unknown.[98]

Dopaminergic neurons of the substantia nigra respond specifically to GDNF, NRTN and BDNF, through GFRα1, GFRα2, and trkB, their respective high-affinity receptors.[103–107] These receptors are located on the cell body and axon terminals of dopaminergic neurons.[104,105,108,109] GDNF can also activate RET via GFRα2 and NRTN via GFRα.[110,111] GDNF can also activate completely different receptors, such as the neural cell-adhesion molecule and syndecan glycoproteins.[112] A large variety of *in vitro* studies have demonstrated that BDNF, NRTN or GDNF play important roles in growth, differentiation and survival of dopaminergic neurons[107,113–115], though the role of these NTFs *in vivo* is not completely understood. GDNF is expressed at low levels in the adult brain, but is indispensable for adult catecholaminergic neuron survival, as shown by the occurrence of massive neuronal death when the GDNF gene is turned off in a conditional knockout mouse model.[116] This finding suggests that if a decrease of GDNF expression occurs in PD, it could contribute to cell death.[117] The role of BDNF in mature dopaminergic neurons is still controversial. Interestingly, BDNF is expressed by dopaminergic neurons.[106,118] Reduced expression of BDNF within the substantia nigra accompanies the deterioration of dopaminergic neurons in PD patients.[119] However, this association is not solidly supported by the results of experiments carried out in BDNF knockout mice. Though a study shows a 23% reduction of these cells at postnatal day 21 in Wnt1-Cre mice,[120] other reports showed that chronic deficits in BDNF alone do not affect survival or function of dopaminergic nigrostriatal neurons during aging, as revealed by the study of the BDNF−/− mice.[121]

The potential utility of GDNF, NRTN and BDNF in promoting the protection and survival of dopaminergic neurons and axonal regeneration is well-established in different animal models of PD.[115,122,123] This question has been studied at length following either intracerebral infusion of a purified recombinant protein or viral gene delivery. Acute or continuous infusions of these NTFs produce robust neuroprotective effects in both rodent

and primate models of PD.[124–126] However, the short half-life of the recombinant protein because of enzymatic degradation *in situ* has encouraged the development of other techniques. Among those described in the literature, thus far, viral vector-mediated gene transfer is undoubtedly the most powerful technique to provide increased levels of transgenic growth factors *in situ*. In laboratory animals, viral vectors can be injected locally in the striatum, in the substantia nigra, or at both locations, either before (preventive) or after (restorative) the administration of the neurotoxic agent. Although an extensive review of the data is beyond the scope of the present review, suffice it to say that in all gene therapy models, viral overexpression of GDNF, NRTN or BDNF have proven effective to prevent the loss nigrostriatal dopaminergic neurons (for reviews, see references# 15,17,127). The success of preclinical studies with infusion protocols and viral gene therapy led to their use in PD patients in an attempt to reduce dopamine neuron degeneration.[128]

To date, only mechanically injected GDNF and viral-delivered NRTN have been explored in clinical trials to cure PD. Whereas they have demonstrated relative safety, they have been clinically disappointing.[19,128,129] The negative findings might have resulted from the fact that only advanced stage PD patients were used for these studies. Because NTFs require that significant dopaminergic innervation remains, earlier-stage PD patients are likely to be the ideal candidates for neurotrophic therapy. Another possibility for the negative results in clinical trials is that the GDNF and NRTN were only supplied within the striatum, without any expression in the cell bodies of dopamine neurons or in their extensive collaterals in other structures. We propose that there is a strong rationale for targeting gene transfer into dopaminergic neurons in the substantia nigra. To evaluate the possibility that the proposed rationale is correct, the NTS-polyplex was used to transfect a neurotrophic gene into dopaminergic neurons of a rat model of PD, as detailed below.[24]

NTS-polyplex-gene therapy model

The 6-OHDA hemiparkinsonian rat model is a useful model to test neurotrophic gene transfection of dopaminergic neurons with the NTS-polyplex (Figure 8). In addition to progressive dopaminergic degeneration, such an animal model of PD avoids additional, mechanical injury of neurons in the substantia nigra. Furthermore, the massive striatal dopamine deficiency causes detectable drug-induced circling behavior at day 7 after 6-OHDA injection.[24] To date, a single dose of NTS-polyplex in the proximity of substantia nigra is the only route of transfection that has been evaluated (Figure 8). A volume of 1 μL of NTS-polyplex dissolved in Dulbecco's modified Eagle medium was injected at a flow rate of 0.1 μL/min. The dosing of NTS-polyplex components was established on the basis of molar ratios determined by retardation and retention microassays by 0.2% agarose gel electrophoresis. [21,22,45] For instance, the molar ratios for NTS-polyplex containing pEF-Bos-hGDNF were determined to be 30 nM pDNA: 20 μM KP: 300 nM neurotensin-carrier (Figure 5). The total amount of pDNA in 1 μL injected was 118 ng, which is significantly lower than the limiting concentration (20 μg/mL) required to form a soluble complex. [37]

Three independent techniques (reverse transcriptase-polymerase chain reaction, immunofluorescence, and western blot) showed that the human GDNF-flag was expressed in the substantia nigra in response to NTS-polyplex transfection (Figure 9).[24] A strong correlation between the reduction of motor impairment and the increase in dopamine content in the substantia nigra and striatum was established. This result suggests that the effectiveness of neurotrophic therapy depends on the recovery of neurotransmission at the level of cell bodies and terminals, which was attained by the intranigral transfection.[24] Moreover, neuron survival in the substantia nigra and striatal reinnervation were also demonstrated, resulting from the presence of human GDNF-flag protein in those nuclei (Figure 10).[24] The presence of human GDNF-flag protein in the striatum (Figure 9, B) strongly suggests the occurrence of both anterograde transport of the human GDNF-flag and

the release of this factor at the level of the neuronal cell body and axon terminals in target structures. All these results support the contention that transfection of the neurotrophic gene into dopaminergic neurons is the correct strategy to simultaneously affect their soma and terminals. Preliminary results of NTS-polyplex-mediated transfection of either NRTN-V5-HIS or BDNF-flag in the 6-OHDA-rat model show neurotrophic and behavioral effects similar to those of the hGDNF-flag transfection.[25,26] Interestingly, NTS-polyplex transfection of NRTN-V5-HIS in a different, chronic model of hemiparkinsonism significantly reduced motor impairment and produced a restorative effect on the dopaminergic nigrostriatal system. In that later study, NTS-polyplex-mediated transfection of NRTN-V5-HIS was performed on day 90 after the 6-OHDA injection, with the behavioral test and TH-immunohistochemistry performed one month after the transfection;[26] this model thus has face validity as a model for assessing treatment of more advanced stage PD.

Together, this evidence suggests that the transfection of neurotrophic genes into dopamine neurons of the substantia nigra is key to achieve simultaneous neurotrophic effects on the remaining soma and terminals. Under such conditions, the expression of GDNF, NRTN and BDNF in the cell body of dopaminergic neurons can be accompanied by localization to axon terminals following anterograde transport.[130] NTFs can then be released from both the soma and axon terminals to exert simultaneous autocrine and paracrine effects on neuronal survival and axon regeneration. Because a single dopaminergic neuron sends extensive collaterals to multiple nuclei in the brain (Figure 1),[5] it is expected that transgenic NTFs will also be present and act upon all of the target structures. This approach is likely to be more effective than the administration of viral vectors or infusions into the caudo-putamen nucleus since this nucleus is much larger than the substantia nigra and several administrations should be required to cover most of the striatal volume and ensure uptake of the NTF by the remaining dopaminergic terminals. Although in principle, striatal expression of NTFs can lead to sufficient supply of these factors to the soma of dopaminergic neurons, this strategy is unlikely to affect the numerous targets of dopamine neurons.

The large extent of dopaminergic denervation and the advanced stage of PD when the therapy is initiated are likely to be important limiting factors for the success of neurotrophic gene-therapy for PD. For an effective gene therapy, we suggest that it may be important to transfect neurotrophic genes into dopaminergic neurons in an early stage of PD in order to take advantage of sufficient substrate for the action of the expressed NTFs.

Towards clinical use of NTS-polyplex

To date, a single injection of the NTS-polyplex in the proximity of the substantia nigra is the only route of neurotrophic gene transfection that has been evaluated in the 6-OHDA hemiparkinsonian-rat model.[24–26] Though studies on the potential neurotoxicity of the NTS-polyplex in the rat have not been done, the results from the local transfection of neurotrophic genes suggest that this approach might also produce attenuation of neuroinflammation caused by 6-OHDA and the surgical procedure.[24–26] In that case, the local administration of the NTS-polyplex in the neurotrophic gene therapy for PD would be a safe and beneficial procedure. In support of this suggestion, clinical trials have shown that neurotrophic factor infusion or injections of NRTN-AAV into the brain have been relatively safe.[19,129] Further support is provided because PD is the only neurodegenerative disorder routinely treated with neurosurgery.[128]

The NTS-polyplex can also deliver a neurotrophic gene from the striatum into nigral dopaminergic neurons through the NTSR1-mediated retrograde transport of NTS.[21] This result suggests that the injection of the NTS-polyplex into the striatum might be another route to transfect dopaminergic neurons in the treatment for PD patients. However, the

striatal injection of the NTS-polyplex has two possible disadvantages in comparison to its nigral injection. The first disadvantage is that the striatum is a much larger nucleus than the substantia nigra in humans. This implies that a large dose of NTS-polyplex or repeated injections of small dose in different sites should be made to cover the whole of the striatum, thus assuring an efficient transfection of the nigral dopaminergic neurons. The second possible disadvantage is the decrease in the NTSR1 density because of the scarcity of dopaminergic axon terminals in the striatum of PD patients that might limit the retrograde transport of the NTS-polyplex to the nigral cell bodies. This is a limiting factor for all forms of neurotrophic gene therapy for PD, especially because of the extensive loss of divergent innervation of multiple structures after degeneration of dopaminergic neurons.

Recently, the NTS-polyplex harboring a suicide gene has been intravenously injected in repeated doses to kill malignant cells in a model of metastatic neuroblastoma developed by subcutaneous allograft of N1E-115 cells in nude mice.[40] This therapeutic approach was based on the finding that neuroblastoma cells overexpress NTSR1.[46] This work showed that the NTS-polyplex was able to reach and transfect only tumorous cells and in a lesser extension the NTSR1-expressing cells of the intestinal tract after the injection into the blood stream.[40] However, no transgene expression was seen in the brain suggesting that the NTS-polyplex is unable to cross the blood brain barrier (BBB), possibly caused by the inability of the NTS to penetrate the BBB. Though the BBB hindrance protects the brain from the NTS-polyplex-mediated transfection of cell-death-promoting genes, the BBB is a limitation in the targeted neurotrophic gene delivery to the brain through the blood stream. Two possible strategies have been considered to overcome the BBB. The first strategy is to replace the NTS as ligand of the NTS-polyplex by its synthetic analogue NT69L, which resists degradation by plasma enzymes, binds NTSR1 with higher affinity than NTS, and is able to cross the BBB.[131] The second invasive strategy involves the systemic administration of the NTS-polyplex in conjunction with transient BBB disruption using vasoactive amines such as bradykinin, histamine, and the synthetic bradykinin analog RMP-7 (receptor-mediated permeabilizer).[132] Other routes of the NTS-polyplex to bypass the BBB might be the intraventricular or intranasal administration, which have been effective in drug delivery into the brain.[132]

Limitations, perspectives, and possibilities of the NTS-polyplex for PD therapy

The NTS-polyplex appears to have a promising future in the neurotrophic gene therapy for PD. However, some limitations unrelated to the nonviral gene technology but with the nature of the disease have raised concerns about the future of this therapeutic approach. First, neuron loss silently accumulates before clinical signs become evident.[1] Symptoms develop when approximately 50% of dopaminergic neurons are still present.[15] Therefore, neurotrophic therapy should start in the early stage of PD to retain sufficient dopamine neurons that are compromised but not dead to halt the advance of neurodegeneration. However, the pharmacological treatment at this stage of PD is still effective and therefore rules out gene therapy as a first-elective approach. Although PD is a gradually developing disease and the potential window of treatment appears to be large, the clinical trials of neurotrophic gene therapy using viral vectors in advanced PD are not conclusive. The other major issue that confronts neurotrophic gene therapy is that it fails to address the etiology and the complexity of PD.[1,4] Though the neurotrophic gene transfection into the compromised dopaminergic neurons might lead to repopulation and restoration of multiple brain circuits in many brain regions (those innervated by those nigral neurons), it fails to repair the genetic alteration that causes Parkinson's disease.[133] Recently, interference RNA technology has been proposed for PD therapy because the over-expression of various proteins is known to kill the nigral dopaminergic neurons in animal models and in familial forms of PD. [41] Similarly to viral vectors, the NTS-polyplex might be used in the delivery of

interference RNA into dopaminergic neurons to knock down the altered proteins, such as α-synuclein or LRRK2 (protein leucine-rich repeat kinase 2).[133,134]

Notwithstanding the limitations that all gene vectors suffer, the NTS-polyplex nanoparticle system seems to have broad perspectives for a clinical use for PD. By taking advantage of the potential of the NTS-polyplex to codeliver two genes into the same cell,[21] this system might be used to deliver a neurotrophic gene and interference RNA. Hypothetically, this approach might yield a neurotrophic effect and decrease in the mutated protein levels. Another possible combined gene therapy using the NTS-polyplex might be with a neurotrophic gene and one of the genes coding for a Redox enzyme such catalase.[135] This treatment would be intended to attenuate neuroinflammation and increase neuroprotection in patients with PD. In summary, a combined gene therapy for PD can be formulated not only with different neurotrophic genes but also with a great variety of genetic approaches, as mentioned above, well beyond the limit of imagination. However, from a theoretical point of view the NTS-polyplex nanoparticles have the limitation to incorporate a drug in addition to the transgene. This incorporation would exceed the precise stoichiometry to assemble the five components of the NTS-polyplex into functional nanoparticles for gene delivery.

Potential use of neurotrophic therapy in other neurodegenerative diseases

An attractive characteristic of the NTS-polyplex that makes it a promising candidate for PD gene-therapy is its specificity through NTSR1-mediated endocytosis mechanism to shuttle the transgene into surviving dopamine neurons. Other neurons of the central nervous system (CNS), such as those of the dopaminergic ventral tegmental area and of the cholinergic basal-forebrain also express NTSR1,[67,136,137] so they are other putative targets for gene delivery via the NTS-polyplex. For example, NTS-polyplex transfection of neurotrophic factors into basal-forebrain cholinergic neurons could be useful to prevent neurodegeneration in Alzheimer's disease.[138,139] Two gene therapy approaches for Alzheimer's disease that have been previously successful with viral vectors could perhaps be adapted to take advantage of NTS-polyplex transfection. The first approach aims to increase NGF concentration in order to promote survival, regeneration and protection of basal-forebrain cholinergic neurons.[139] The second approach is overexpression of the CREB-binding protein to overcome the interference of amyloid-β accumulation, which plays a primary role in the cognitive deficits of AD.[140]

Although the potential of neurotrophic gene-therapy is very extensive, the absence of NTSR1 in degenerating neurons limits the use of NTS-polyplex in other pathologies of the central and peripheral nervous system.

Benefits and limitations of NTS-polyplex system in comparison to viral carriers

Polyplexes have the advantage over viral gene transfer of being non-immunogenic, easy to produce and not oncogenic. Although the immunological and safety evaluation of the NTS-polyplex has not been accomplished, published evidence, both direct and indirect, supports the biosafety of NTS-polyplex-mediated transfection *in vitro* and *in vivo*. A recent study reports that NTS-polyplex-mediated transfection does not affect cell viability *in vitro*.[21] In all of the studies performed *in vivo*, the NTS-polyplex has been shown not to cause more damage to brain parenchyma than that caused by mechanical microinjection by itself.[20–22,24–26] Moreover, repeated intravenous injections of NTS-polyplex for targeted gene-delivery of a suicide gene in an animal model of neuroblastoma lead to an antitumoral effect without signs of tissue damage in other organs.[40] This evidence agrees with the demonstrated biosafety of lactoferrin-polyplex, that has been successfully used in animal models of PD.[141,142] In contrast, non-degradable polyplexes, such as those based on high molecular weight polyethylenimines, are able to cause cytotoxicity as a result from their

poor clearance.[143] These later kinds of polyplexes have limited use in gene therapy despite their high transfection efficiency *in vitro* and *in vivo*.

To date, only lactoferrin-polyplex and NTS-polyplex systems have been explored to deliver the GDNF gene delivery in parkinsoninan rats.[24,144] The efficacy of those systems in promoting functional recovery in the rat 6-HODA lesion model is similar to that of viral carriers. [145,146] However, clinical trials with such nanoparticles will be required to confirm the usefulness of this strategy for the treatment of PD.

Lack of regulation of gene expression is a current limitation for all current protocols in neurotrophic therapy. The uncontrolled and robust expression of a neurotrophic protein might lead to undesirable side effects such as aberrant innervation of the regenerating dopaminergic fibers [147] and decrease of dopamine synthesis.[148] Furthermore, neurotrophins, for instance, could reach levels high enough to stimulate the p75 receptor, thus leading healthy and recovering neurons to apoptosis.[149] The use of inducible promoters in conjunction with NTS-polyplex thus needs to be explored.

Conclusions

Neurotrophic gene-therapy continues to be at the forefront of PD research because it holds the promise of stopping neurodegeneration, reversing neurological damage and maintaining the integrity of divergent projections of nigral dopaminergic-neurons. All neurotrophic gene-therapy approaches are effective and relatively safe in rodent and primate models of PD, although some of them, in particular those with GDNF, produce undesirable behavioral effects resulting from aberrant innervation of the regenerating dopaminergic fibers[147] and decrease of dopamine synthesis.[148] Although the results of clinical trials with recombinant GDNF-protein infusion or NRTN gene-therapy have not been particularly positive, they show that these approaches are safe in humans. We believe that the modest clinical benefit might be related to the selection of putamen cells as the source of NTFs. From this region, the diffusion of NTFs confronts physical and biochemical obstacles to reach the cell bodies and the other innervation targets of dopaminergic neurons. In particular, the long distance that separates the other targets from the putamen in the human brain and enzymatic degradation of NTFs could be the main causes of the poor neurotrophic effect. We propose that transfection of neurotrophic genes in dopaminergic neurons might be a much more effective strategy to obtain a neurotrophic effect simultaneously in the cell body and terminal regions of dopaminergic neurons. The NTS-polyplex has the ability to transfect dopaminergic neurons via NTSR1-mediated endocytosis at the cell body or via retrograde transport of NTSR1. Nigral injection of the NTS-polyplex with neurotrophic genes (GDNF, NRTN or BDNF) has been effective in the treatment of acute and chronic experimental hemiparkinsonism in the rat. The preliminary data suggesting adequate biosafety of this approach anticipates the safe use of the NTS-polyplex in clinical trials of neurotrophic gene-therapy for PD in the near future. Meanwhile, evaluation of the NTS-polyplex in Parkinsonian nonhuman primates represents the next important test of this promising nonviral gene-transfer system.

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List of abbreviations

AAV	adeno-associated virus
ARTN	artemin
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
sCDNF	cerebral dopamine-neurotrophic factor
CMV	cytomegalovirus
CNS	central nervous system
DAT	dopamine transporter
FP	fusogenic peptide
GDNF	glial cell-line-derived neurotrophic factor
GFLs	glial cell-line-derived neurotrophic factor family ligands
GFP	green fluorescence protein
GFR	high-affinity receptors for glial cell-line-derived neurotrophic factor family ligands
IL	interleukin
KP	karyophilic peptide
MANF	mesencephalic astrocyte-derived neurotrophic factor
NGF	Nerve growth factor
NLS	nuclear-location signal
NRTN	neurturin
NT	neurotrophin
NTF	neurotrophic factor
NTS	neurotensin
NTSR	neurotensin receptor
6-OHDA	6-hydroxydopamine
PD	Parkinson’s disease
pDNA	plasmid deoxyribonucleic acid
PSPN	persephin
SV40	simian virus 40
TH	tyrosine hydroxylase
Trk	tyrosine kinase receptor

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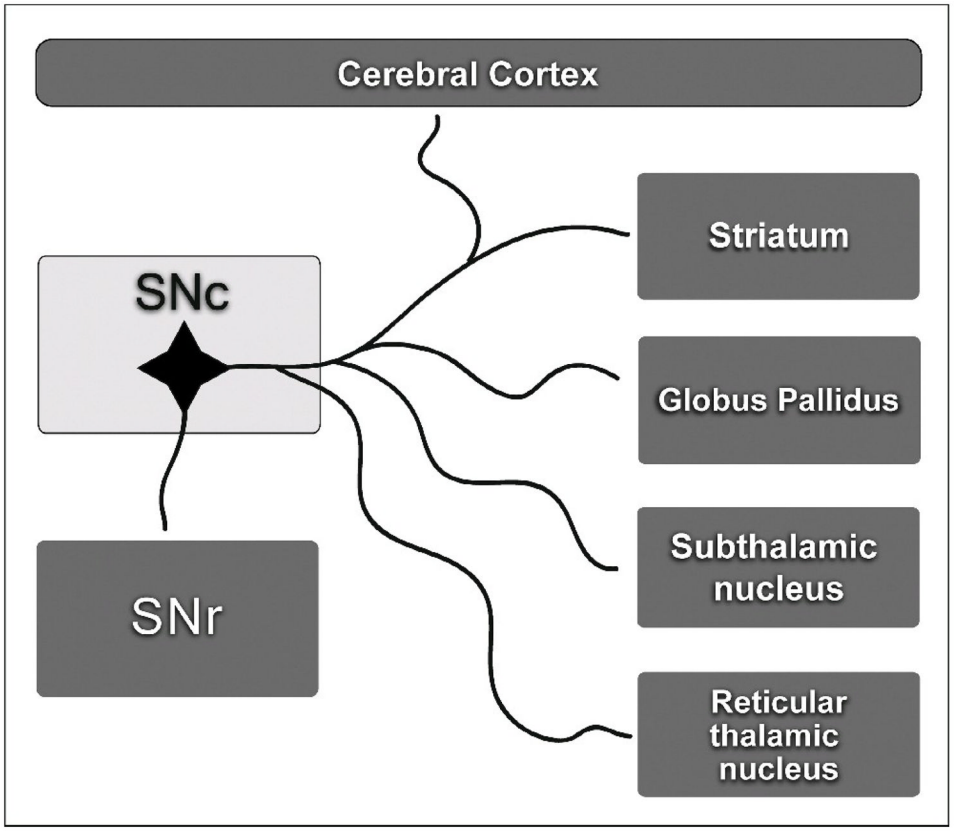

Figure 1.
Diagram showing that the branching of dopaminergic axons originating in the SNc is quite extensive, innervating several nuclei of the brain. The lesion of dopaminergic neuron cell bodies can thus reduce dopamine transmission in multiple structures, thus causing the motor and nonmotor manifestations of Parkinson's disease.

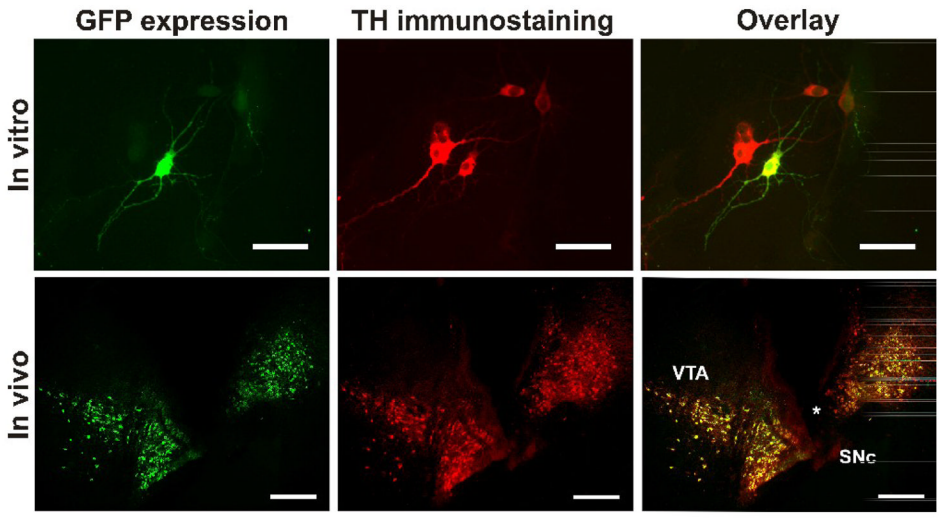

Figure 2.

Ability of NTS-polyplex to transfect dopaminergic neurons in vitro and in vivo. Top panel. Confocal micrographs showing the GFP expression in cultured dopaminergic neurons after transfection with the plasmid pEGFP-N1. NTS-polyplex was formed at 1:833:36 molar ratio (pEGFP-N1, 6 nM: PK, 5 μM: NTS-vector, 216 nM) in a mixture of Neurobasal medium and B27, serum free, and applied immediately to 2-day-old primary cultures. After 6-h exposure, the transfection medium was replaced by fresh culture medium and cells were incubated for an additional 72 h, as described previously. [150] Cells were subjected to double immunofluorescence against GFP and TH. The primary antibodies were a rabbit polyclonal antibody to GFP (Abcam; Cambridge MA, USA) and a mouse monoclonal antibody to TH (Sigma-Aldrich, St. Louis, MO, USA). The secondary antibodies were an Alexa 488 chicken anti-rabbit (Molecular Probes Inc., Eugene, OR, USA) and a donkey anti-mouse TRITC (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). Calibration bars = 50 μm. Bottom panel. Confocal micrographs showing the GFP expression in dopaminergic neurons of the substantia nigra after the local injection of the NTS-polyplex with pDAT-EGFP. Calibration bars = 200 μm. Micrographs of panel B were reproduced from Arango-Rodriguez et al. (2006).

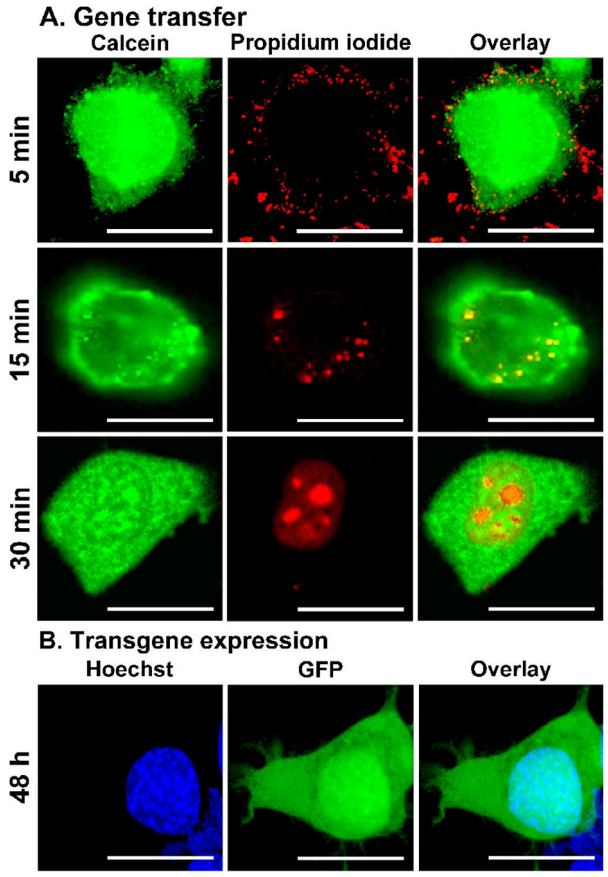

Figure 3.

Schematic illustration of the sequence of NTS-polyplex-mediated gene transfection documented by confocal microscopy in neuroblastoma N1E-115 cells. A. NTS-polyplex harboring a propidium iodide-labeled plasmid DNA was used for transfection and calcein was used to delimit the cell area. The micrographs of the first row were taken 5 min after incubation with propidium iodide-labeled NTS-polyplex in the presence of 0.45 M sucrose to block receptor-mediated endocytosis [58]. The incubation time with 0.45 M sucrose was 30 min.[22,27,45] B. GFP expression in N1E-115 cells counterstained with Hoechst, a nuclear staining. The microphotographs were taken at the times after transfection shown at the left margins. Calibration bars = 20 μm. The transfection efficiency is shown in figures 6 and 7 (in vitro) and figures 2 and 9 (in vivo). The first and third panels were reproduced from Navarro-Quiroga et al. (2002).

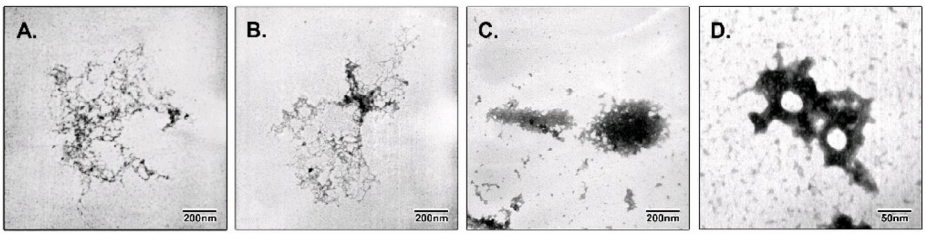

Figure 4.

Electron microscopy analysis of the sequential steps of the formation of the NTS-polyplex nanoparticles with pEGFP-N1. The micrographs show the natural form of 6 nM pDNA alone (A), the initial condensation of pDNA caused by the addition of 6 μM KP (B), the condensation of the KP-pDNA complex in the presence of 1% FBS (C), and the toroidal condensation of the KP-pDNA complex caused by the addition of the NTS-vector at the optimum molar ratio (1:34) to form the NTS-polyplex nanoparticles (D). Micrographs were reproduced from Arango-Rodriguez et al. (2006).

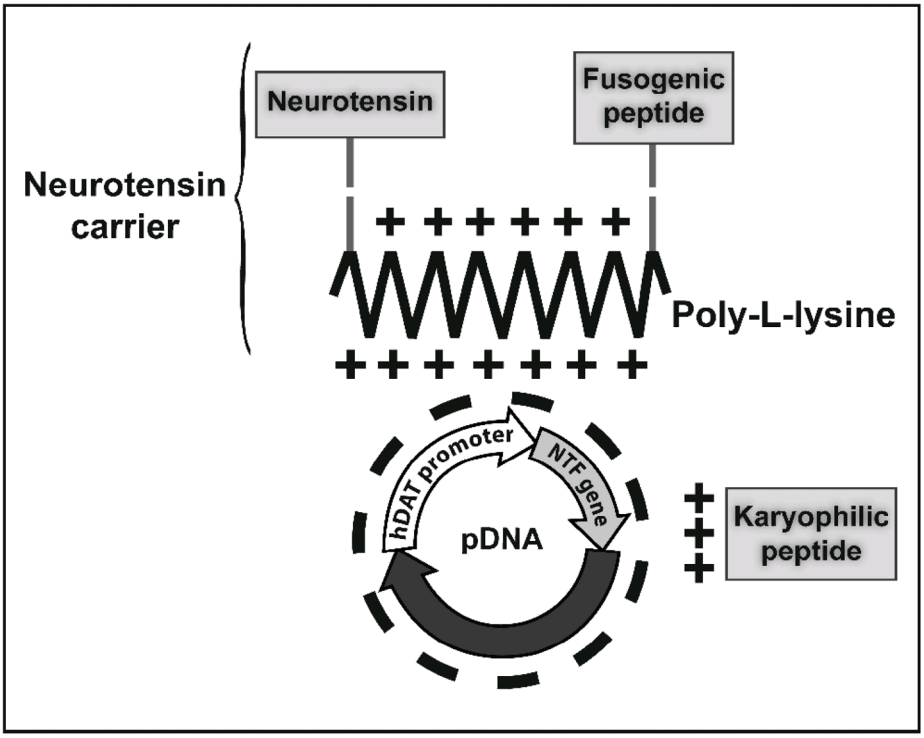

Figure 5.

Scheme of the NTS-polyplex components. The neurotensin carrier is the conjugate of neurotensin and fusogenic peptide with poly-L-lysine. The karyophilic peptide is electrostatically bound to a plasmid DNA (pDNA) to form the KP-pDNA complex, which is bound to the neurotensin carrier to form the NTS-polyplex. The pDNA encompasses the gene of interest under control of a tissue-specific promoter; for instance, hDAT (human dopamine transporter), a promoter specific for dopaminergic neurons. NTS = pyroGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH. Modified hemagglutinin HA2 FP = Gly-Leu-Phe-Glu-Ala-Ile-Ala-Glu-Phe-Ile-Glu-Gly-Gly-Trp-Glu-Gly-Leu-Ile-Glu-Gly-Cys-Ala-Lys-Lys-Lys-OH. SV40 Vp1 NLS = Met-Ala-Pro-Thr-Lys-Arg-Lys-Gly-Ser-Cys-Pro-Gly-Ala-Ala-Pro-Asn-Lys-Pro-Lys-OH.

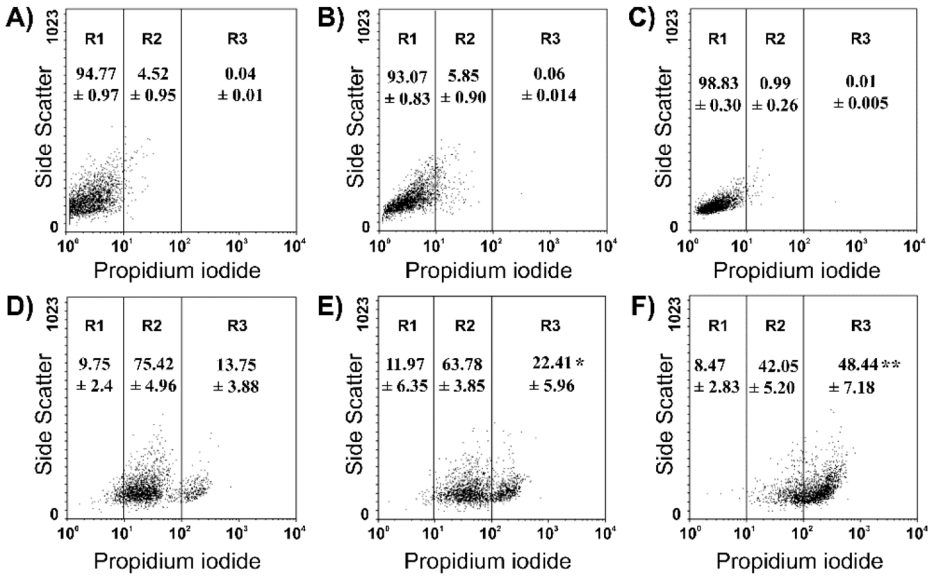

Figure 6.

Flow cytometry analysis showing the contribution of fusogenic and karyophilic peptides to the improvement of NTS-polyplex internalization. The plasmid was labeled with propidium iodide. (A) Basal fluorescence in N1E-115 cells.(B) Blockade by 100 nM SR-48692 of fusogenic-karyophilic-NTS-polyplex internalization in N1E-115 cells. (C) Failure of fusogenic-karyophilic-NTS-polyplex to internalize in COS7 cells, which lack NTSR1. (D) Internalization of karyophilic-NTS-polyplex in N1E-115 cells.(E) Internalization of fusogenic-NTS-polyplex in N1E-115 cells.(F) Internalization of fusogenic-karyophilic-NTS-polyplex in N1E-115 cells. Populations of 10^4 cells emitting red fluorescence of propidium iodide were distributed in three arbitrary regions (R1, R2 and R3) according to their fluorescence intensity. *, Significantly different from R3 in (D); **, significantly different from R3 in (E). The figure was reproduced from Navarro-Quiroga et al. (2002).

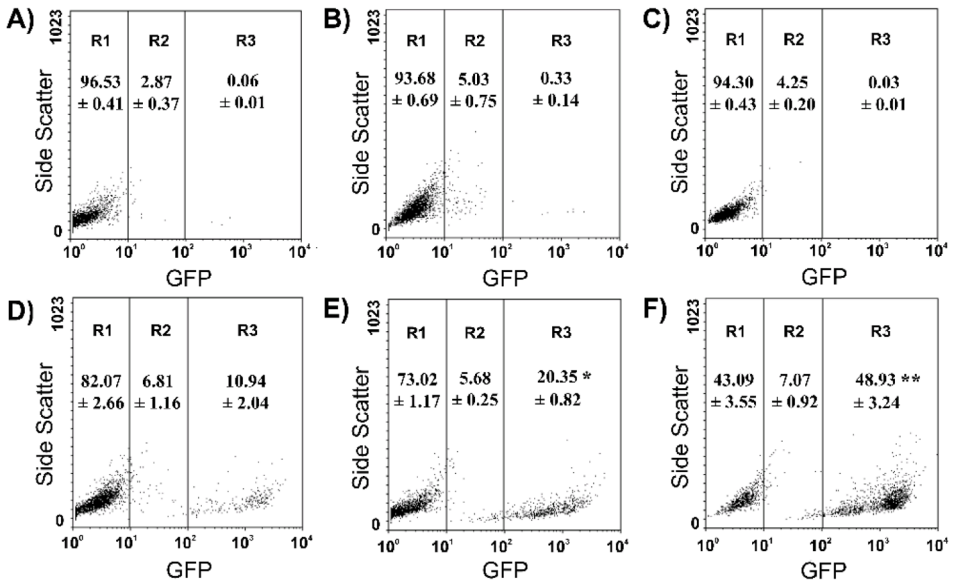

Figure 7.

Flow cytometry analysis showing the contribution of fusogenic and karyophilic peptides to the improvement of NTS-polyplex-mediated gene expression. pGreen Lantern 1 was delivered by different polypplexes. (A) Basal fluorescence in N1E-115 cells. (B) Lack of GFP expression in N1E-115 cells exposed to the fusogenic-karyophilic-NTS-polyplex in the presence of 100 nM SR-48692. (C) Lack of GFP expression in COS7 exposed to fusogenic-karyophilic-NTS-polyplex. (D) GFP expression in N1E-115 cells transfected by karyophilic-NTS-polyplex. (E) GFP expression in N1E-115 cells transfected by karyophilic-NTS-polyplex fusogenic-NTS-polyplex. (F) GFP expression in N1E-115 cells transfected by karyophilic-NTS-polyplex fusogenic-karyophilic-NTS-polyplex. Populations of 10^4 cells emitting the GFP fluorescence were distributed in three arbitrary regions (R1, R2 and R3) according to their fluorescence intensity. *, Significantly different from R3 in (D); **, significantly different from R3 in (E). The figure was reproduced from Navarro-Quiroga et al. (2002).

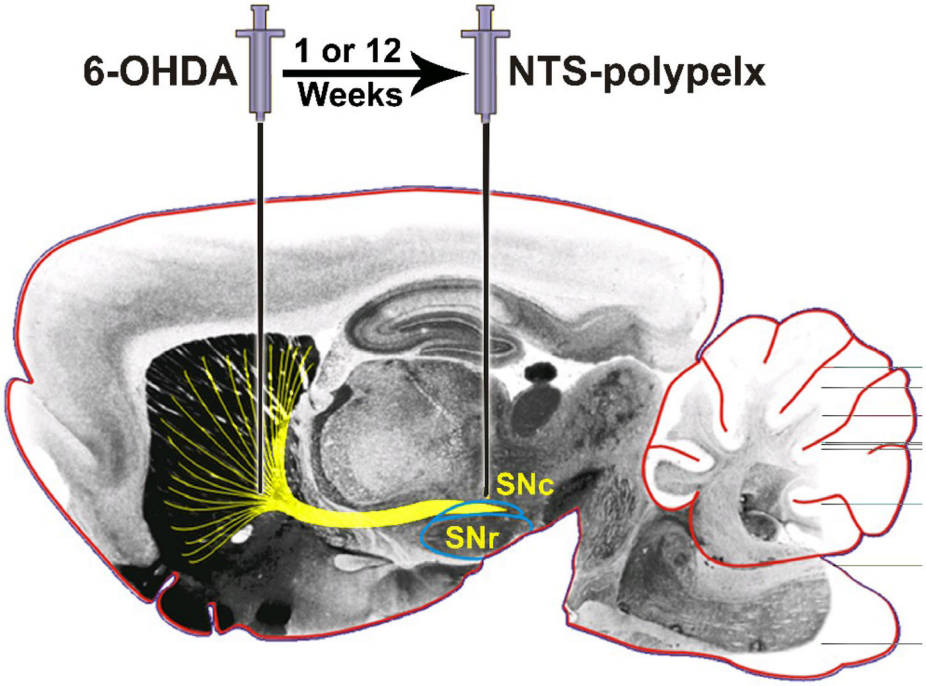

Figure 8.

Sites of the 6-OHDA lesion and NTS-polyplex transfection. 6-OHDA was injected in the striatum at the coordinates: anteroposterior (AP) +7.7 mm from interaural line; mediolateral (ML) + 4.0 mm from interparietal suture and dorsoventral (DV) −5.4 mm from dura mater. One or twelve weeks after 6-OHDA injection, different NTS-polyplexes were injected into the ipsilateral substantia nigra at the coordinates AP + 2.5 mm from interaural line, ML + 2.0 mm from midline and DV −6.7 from dura mater. The coordinates were adapted from the Paxinos atlas for rats weighing 220 g. [24]

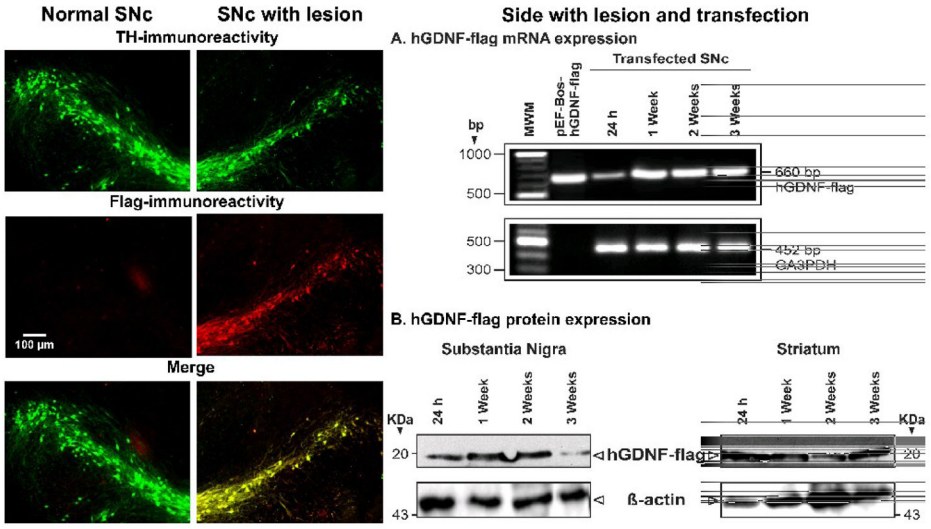

Figure 9.

The overexpression of hGDFN gene in dopaminergic neurons of the substantia nigra leads to the presence of hGDFN protein in the striatum, suggesting the axonal transport of the transgenic protein to the other nuclei innervated by nigral dopaminergic neurons. The micrographs of double immunofluorescence against TH and flag show hGDNF-flag expression in dopamine neurons of the substantia nigra of hemiparkinsonian rats at 3 weeks following intranigral transfection of pEF-BoshGDNF-flag using NTS-polyplex. The calibration bar in the left middle panel is valid for all other panels. A) The representative RT-PCR assays shows the time course of hGDNF-flag gene expression in the substantia nigra of hemiparkinsonian rats. B) The western blot assay shows the presence of hGDNF-flag protein in the substantia nigra and striatum. The figures were reproduced from Gonzalez-Barrios et al. (2006).

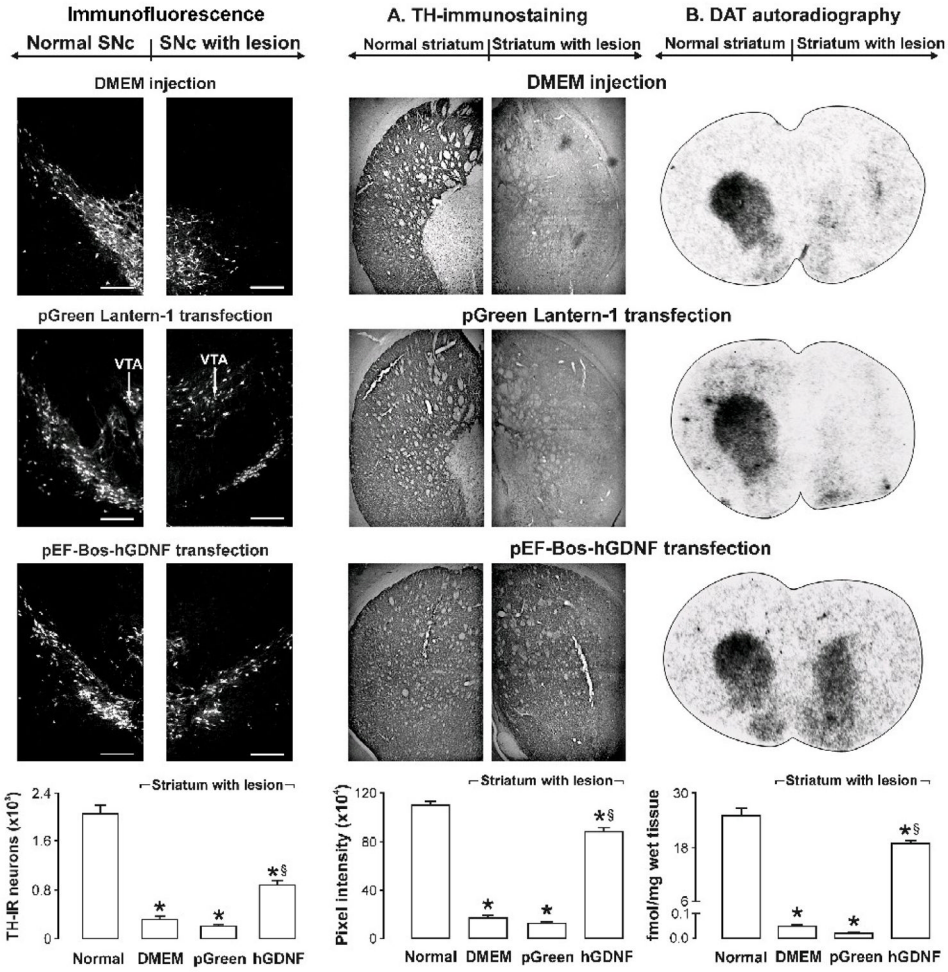

Figure 10.

pEF-Bos-hGDNF transfection in the substantia nigra promotes survival in the nigral dopaminergic neurons and reinnervation of the striatum in hemiparkinsonian rats. The figures were reproduced from Gonzalez-Barrios et al. (2006).