Repairing the parkinsonian brain with neurotrophic factors

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No therapy exists to slow down or prevent Parkinson’s disease (PD), a debilitating neurodegenerative disorder. Neurotrophic factors (NTFs) emerged as promising disease-modifying agents in PD and are currently under clinical development. We argue that efforts in three research areas must converge to harness the full therapeutic power of NTFs. First, the physiological roles of NTFs in aging dopaminergic neurons must be comprehensively understood. Second, the mechanisms underlying the neuroprotective, neurorestorative and stimulatory effects of NTFs on diseased neurons need to be defined. Third, improved brain delivery of NTFs and new ways to stimulate NTF signaling are required to achieve clinical benefits. In this review, we discuss progress in these areas and highlight emerging concepts in NTF biology and therapy.

Introduction

More than five million people are afflicted with Parkinson’s disease (PD) worldwide. The incidence of PD increases with age and the number of cases is expected to increase steadily, paralleling the aging of human society. The degeneration of midbrain dopaminergic neurons in the substantia nigra (SN) and the subsequent depletion of dopamine in the striatum is the major pathological event in PD. A second pathological correlate of PD is the presence of cytoplasmic aggregates (called Lewy Bodies) containing α-syn in the remaining SN neurons [1]. Neuropathology in PD is widespread, extending well beyond the nigro-striatal system (NSS), and progressively affecting other neuronal populations [2]. Current understanding of PD is incomplete and, although studies of genes associated with familial PD (see Glossary) have identified several pathogenic processes that might cause disease [1], there is a lack of an animal model that recapitulates all disease features. The inability of currently available symptomatic therapies (based on dopamine replacement) to prevent, delay, or stop the disease is perhaps the most frustrating aspect of PD.

Neurotrophic factors (NTFs) are secreted proteins that regulate the development, maintenance, function and plasticity of the vertebrate nervous system. Four major classes of molecules comprise the NTF family: (i) the neurotrophin family; (ii) the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs); (iii) the neurotrophic cytokines (neurokines); and (iv) the new family of cerebral astrocyte-derived neurotrophic factor (MANF) (Table 1). NTFs are the most potent mediators of neuronal survival

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**Glossary**

*Adeno-associated virus (AAV):* a small virus that infects dividing and non-dividing cells. AAV transduces neurons with high efficiency, but it does not induce strong immunological responses. AAV might integrate into the genome, but only at specific sites. The AAV2 serotype is the best studied and the only one utilized in clinical trials so far.

*Double-blind, placebo-controlled trial:* a type of clinical trial in which neither the researchers nor the participants know whether a placebo (inert tablet or sham surgery) or the active compound is being administered.

*Double-blind, placebo-controlled, delayed-start trial (DBDST):* a trial specifically designed to detect potential disease-modifying effects (as opposed to symptomatic improvement). A first phase, in which participants receive either the active compound or placebo, is followed by a second phase, in which all participants receive the active compound. Persistent differences between treatment with active compound versus placebo after the second phase cannot be explained by symptomatic relief only, and suggest the existence of disease-modifying effects.

*Familial PD:* (as opposed to sporadic PD) represents a subset (~5–10%) of PD patients with a family history of PD. Mutations in five genes have been consistently associated with familial PD cases. Mutations in genes encoding α-syn and leucine-rich repeat kinase 2 (LRRK2, also called dardarin) cause autosomal dominant PD, whereas mutations in those encoding parkin, DJ-1 and PINK1 cause autosomal recessive PD. Other genes (including those encoding ATPase type 13A2 (ATP13A2) and the HTRA serine peptidase 2 (HTRA2, also called OM1)) have been associated with PD or might act as risk factors for PD, but the evidence is currently weak.

*6-hydroxydopamine (6-OHDA):* a dopaminergic- and noradrenergic-specific neurotoxin that cannot pass the BBB and must be injected locally. Re-uptake transporters enable 6-OHDA to enter dopaminergic neurons, where it generates free radicals and leads to cell death.

*Lentivirus (LV):* a retrovirus that infects both dividing and non-dividing cells. LVs are effective in infecting neurons and do not induce immune responses. However, they do integrate into the host genome at unpredictable sites and thus might be a cause of concern because of their oncogenic potential. LVs have been extensively utilized for *ex vivo* gene transfer.

*1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP):* a dopaminergic-specific neurotoxin that can pass the BBB. MPTP is metabolized by glial cells to release MPP⁺, which is transported inside dopaminergic neurons and blocks mitochondrial complex I, leading to cell death.

*Neuroprotective agents:* compounds that are able to prevent or slow down neurodegeneration. This is tested experimentally by applying the agent before the injury and determining whether this decreases the extent of neurodegeneration.

*Neurorestorative agents:* compounds that are able to repair already damaged neurons (e.g. by promoting axonal repair) and might also promote their survival. This effect is tested experimentally by applying the agent after the injury and determining whether this can improve neuron function and/or survival.

*Open label trial (open trial; OL):* a type of clinical trial in which both the researchers and the participants know which treatment is being administered. An OL might still be randomized (i.e. the treatments or conditions are randomly allocated).

*Rotenone:* a widely used insecticide that, at higher doses, induces Parkinsonism in laboratory animals. Rotenone can pass the BBB and accumulates in many types of neurons, where it inhibits mitochondrial complex I activity. Dopaminergic neurons are particularly vulnerable to its toxic effects.

*Stimulatory agents:* promote neuronal metabolism and activity, including the release of neurotransmitter.
identified to date and, not surprisingly, have emerged as promising therapeutic agents for neurodegenerative disorders, including PD. Fuelled by encouraging results in cellular and animal models of PD (Table 1), several clinical orders, including PD. Fuelled by encouraging results in promising therapeutic agents for neurodegenerative disorders, including PD. Fuelled by encouraging results in cellular and animal models of PD (Table 1), several clinical trials (discussed below) have involved the administration of GDNF to PD patients. The conflicting results obtained are perhaps not surprising, given the current rudimentary understanding of the roles of NTF in aging and diseased DA neurons. A successful NTF therapy for PD will require an in-depth molecular and integrative understanding of the impact of NTFs on physiological and pathophysiological processes at play in dopaminergic neurons, coupled with effective and safe strategies for activating NTF signaling in the human brain. Here, we present new developments in these basic, pre-clinical and clinical areas of NTF biology and therapy.

Physiological roles of NTFs in dopaminergic neurons
Whereas the pharmacological properties of many NTFs have been tested on diseased DA neurons (Table 1), comparatively little is known about their physiological roles in intact and aging neurons. We discuss here the physiology of GDNF and the new CDNF/MANF factors, the most potent pharmacological agents in experimental studies of PD.

GDNF
Identified in 1993 as a survival-promoting agent for cultured dopaminergic neurons [3], GDNF also maintains motor, sympathetic, parasympathetic, sensory and enteric neurons; outside the nervous system, it regulates kidney development and spermatogenesis [4,5]. GDNF is the prototypical member of the GFLs, which also comprise neurturin (NRTN), artemin (ARTN) and persephin (PSPN); the latter two factors lack co-receptors in the central nervous system (CNS) and act only in the periphery. Signal transduction by GFLs is unique among NTFs and involves the initial interaction between a dimeric GFL and two molecules of GDNF family receptor α (GFRα1–4) co-receptor (Figure 1). The ensuing GFL–GFRα complex recruits the Ret receptor tyrosine kinase, the common signaling receptor for all four GFLs. Formation of the tripartite GFL–GFRα–Ret complex promotes Ret dimerization and reciprocal trans-autophosphorylation within the Ret intracellular tyrosine kinase domain [4]. The GFRα1–Ret complex can also form in the absence of GDNF, thus anticipating a complex regulation of Ret function [6]. Phosphorylated tyrosine residues of Ret serve as anchoring points for adaptor proteins that initiate several signaling pathways (Figure 1). A second signaling mode for GDNF is via the alternative receptor, the neural cell adhesion molecule (NCAM); although NCAM can directly bind GDNF, the presence of the GFRα1 co-receptor is required for high-affinity binding and activation of downstream signaling [7]. Formation of the GDNF–GFRα1–NCAM complex leads to activation of focal adhesion kinase (FAK) and Fyn kinase, which control neuronal migration [5]. The existence of yet unidentified additional receptor(s) for GDNF has been postulated [8]. A unique property of GDNF, observed in hippocampal neurons, is to promote synaptogenesis by interacting with GFRα1 and promoting GFRα1-mediated homophilic cell–cell interactions [9]. The relevance of this novel ligand-induced cell adhesion system for dopaminergic synapses is currently unknown.

Compelling genetic evidence suggests a physiological role for GDNF in the maintenance and function of the aging NSS. Aging (12–20-month-old) mice heterozygous

<table>
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a Abbreviations: NT-3, neurotrophin-3; NT-4, neurotrophin-4; CNTF, ciliary neurotrophic factor; LIF, leukemia-inhibitory factor; IL-6, interleukin-6; CT-1, cardiotrophin-1; OSM, oncostatin-M; bFGF, basic fibroblast growth factor; ND, not determined.

b See main text.

c Several other factors (including insulin-like growth factor 1 (IGF-1); epidermal growth factor (EGF); vascular endothelial growth factor (VEGF); vasoactive intestinal peptide (VIP); erythropoietin (EPO); sonic hedgehog (SHH) and bone morphogenetic proteins (BMPs)) were reported to exert effects on dopaminergic neurons (reviewed in [23,27,91,93]).
for either GDNF [10] or GFRα1 [11] display a subtle loss of neurons in the SN. Knock-in mice carrying constitutively active Ret (Ret\textsuperscript{MEN2B}) exhibit an increase (26%) in SN [but not in ventral tegmental area (VTA)] neurons and enhanced levels of striatal dopamine, indicating that Ret has a potent effect on SN neurons [12]. The analysis of animals completely lacking GDNF/Ret signaling components has been complicated by the early lethality associated with constitutive inactivation of GDNF, GFRα1or Ret [5]. To circumvent this early lethality, GDNF function has been constitutively removed from early adulthood onwards, using tamoxifen-induced Esr1-Cre-mediated excision. Although GDNF expression in the brain was decreased by only 60%, a severe degeneration of SN and VTA dopaminergic neurons and of locus coeruleus (LC) noradrenergic neurons was found after 5 months of age, which was associated with progressive hypokinesia [13]. One study in which Ret was ablated during embryonic

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**Figure 1.** Overview of GDNF signaling. (a) Upper left: GDNF signaling via the Ret receptor tyrosine kinase involves the initial interaction between GDNF and membrane-bound GFRα1 (located in lipid rafts). The GDNF–GFRα1 complex recruits Ret into lipid rafts and potentiates Ret downstream signaling. Upper right: an alternative mode for activation of Ret is via soluble GFRα1 (sGFRα1), which binds GDNF and activates Ret in trans (initially outside lipid rafts). Middle: activated Ret initiates several signaling pathways, including Ras–ERK, PI3K–Akt, Src and PLC–PKC, which regulate diverse processes, including neuron survival. PI3K–Akt and Ras–ERK signaling might promote survival by phosphorylating several downstream targets, including transcription factors [p53, cAMP response element binding protein (CREB) and forkhead box O (FOXO)], translation factor inhibitors (e.g. bad, c-Myc), and pro-apoptotic proteins (e.g. caspases, BAD and glycogen synthase kinase-3 (GSK3)); these targets have not been validated in dopaminergic neurons. Several proteins [including the insulin receptor substrate 1/2 (IRS1/2); downstream of kinase 1/4/5 (Dock1/4/5); signal transducer and activator of transcription 3 (STAT3) or Enigma] are also recruited downstream of activated Ret, but the outcomes of these interactions are poorly understood. It remains to be determined whether all or a subset of these interactions promote survival of dopaminergic neurons. (b) Regulation of neuronal survival by GDNF involves not only local signaling (at axon terminals), but also long-range signaling, via retrograde transport from axon terminals into the cell body. The GDNF–Ret complex is endocytosed and the ensuing signaling endosomes (pink dots on the left and enlarged view on the right), which carry numerous signaling components, are anchored to the microtubule network (by anchoring proteins such as dynein–dynactin) and transported towards the soma.

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**Diagram:**

- **Microtubule network**
  - **Survival oxidative stress response**
- **Lower left:** GDNF signaling via the Ret receptor tyrosine kinase involves the initial interaction between GDNF and membrane-bound GFRα1 (located in lipid rafts). The GDNF–GFRα1 complex recruits Ret into lipid rafts and potentiates Ret downstream signaling. Upper right: an alternative mode for activation of Ret is via soluble GFRα1 (sGFRα1), which binds GDNF and activates Ret in trans (initially outside lipid rafts). Middle: activated Ret initiates several signaling pathways, including Ras–ERK, PI3K–Akt, Src and PLC–PKC, which regulate diverse processes, including neuron survival. PI3K–Akt and Ras–ERK signaling might promote survival by phosphorylating several downstream targets, including transcription factors [p53, cAMP response element binding protein (CREB) and forkhead box O (FOXO)], translation factor inhibitors (e.g. bad, c-Myc), and pro-apoptotic proteins (e.g. caspases, BAD and glycogen synthase kinase-3 (GSK3)); these targets have not been validated in dopaminergic neurons. Several proteins [including the insulin receptor substrate 1/2 (IRS1/2); downstream of kinase 1/4/5 (Dock1/4/5); signal transducer and activator of transcription 3 (STAT3) or Enigma] are also recruited downstream of activated Ret, but the outcomes of these interactions are poorly understood. It remains to be determined whether all or a subset of these interactions promote survival of dopaminergic neurons. (b) Regulation of neuronal survival by GDNF involves not only local signaling (at axon terminals), but also long-range signaling, via retrograde transport from axon terminals into the cell body. The GDNF–Ret complex is endocytosed and the ensuing signaling endosomes (pink dots on the left and enlarged view on the right), which carry numerous signaling components, are anchored to the microtubule network (by anchoring proteins such as dynein–dynactin) and transported towards the soma.

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**Diagram (continued):**

- **Survival oxidative stress response**
- **Retrograde signaling**
  - **Signaling endosome**
    - **Lipid bilayer**
  - **Microtubule network**

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**Diagram (additional details):**

- **GDNF**
  - **GFRα1**
  - **Ret**
  - **PI3K**
  - **Akt**
  - **ERK**
  - **Ras**

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**Diagram (additional details):**

- **Survival oxidative stress response**
  - **Pre-synaptic cell**
  - **Post-synaptic cell**
  - **GDNF-induced cell adhesion**
  - **Microtubule network**

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**Diagram (additional details):**

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  - **Pre-synaptic cell**
  - **Post-synaptic cell**
  - **GDNF-induced cell adhesion**
  - **Microtubule network**
development using a dopaminergic-specific Cre line found no neurodegenerative signs in 8–12-month-old mice [14]. In a more recent study using a similar approach, a moderate degeneration in the SN and a marked degeneration of dopaminergic nerve terminals in the striatum of 12–24-month-old animals were observed [15]. These findings establish Ret as a crucial regulator of long-term maintenance of the NSS. They also suggest that: (i) GDNF signals via its alternative receptor NCAM to promote survival of SN, VTA and/or LC neurons; and (ii) embryonic inactivation of Ret induces compensatory changes in dopaminergic neurons that are not triggered upon the removal of GDNF function at a later developmental time point.

It is currently unclear how GDNF-deprived SN neurons succumb to neurodegeneration. Results obtained with cultured embryonic dopaminergic neurons deprived of GDNF indicate that these neurons die via a death receptor- and caspase-dependent, but mitochondria-independent, pathway [16], suggesting that GDNF promotes neuronal survival by inhibiting the death receptor pathway. The molecular mechanisms underlying the pro-survival function of GDNF in aging SN neurons are difficult to address in vivo, partly because of practical restrictions imposed by the aging process. In cultured neurons, GDNF, similar to other NTFs, activates several pro-survival pathways, including the phosphatidylinositol-3-kinase (PI3K)/Akt, Ras/extracellular-signal-regulated kinase (ERK), Src kinase and phospholipase C-γ (PLC-γ) pathways (Figure 1) [4,5]. Full activation of Ret requires its translocation into lipid rafts, which promote its stability by preventing its proteasomal degradation [17]. Recent evidence suggests that proteasomal degradation of Ret in distal axons regulates the survival capacity of GDNF by modulating the amount of active GDNF–GFRA1–Ret complexes that are retrogradely transported to cell bodies [18]. Another way to regulate Ret signaling is via the Sprouty family of proteins, which act at several levels to inhibit Ret activation and downstream signaling [5]. The transmembrane leucine-rich-repeats and immunoglobulin-like domains 1 (Lrig1) protein physically interacts with Ret and inhibits its function in motor and sympathetic neurons, although its interaction with Ret in dopaminergic neurons has not yet been investigated [19]. GFRA1 is also regulated by endocytosis [20] and by phosphatidylinositol-specific phospholipase-C, which cleaves off the glycosylphosphatidylinositol (GPI) anchor to release soluble GFRA1 [5]. Targeting these endogenous mechanisms that modulate GDNF/Ret signaling might be of therapeutic interest in relation to diseased dopaminergic neurons.

**CDNF/MANF**

CDNF is a novel trophic factor for dopaminergic neurons that has recently been identified [21]. It is similar to the previously identified MANF [22] and both factors are expressed in the mouse NSS [23]. Human CDNF and MANF share 59% sequence homology and CDNF/MANF homologues have also been identified in *Drosophila* and *Caenorhabditis elegans* [23]. The *Drosophila* ortholog, DmMANF, is important for the maintenance of dopaminergic-positive neurites and dopamine levels, and its inactivation causes non-apoptotic cell death during late embryonic development [24]. An interesting feature of the CDNF/MANF family is that it lacks survival-promoting activities in peripheral sympathetic and sensory neurons in culture, in contrast to all other NTFs [23]. CDNF and MANF are structurally unrelated to all known NTFs, suggesting a novel mechanism of action. Besides being secreted, these factors are retained within the endoplasmic reticulum (ER), where they might assist protein folding and mitigate ER stress, thereby preventing neurodegeneration [23]. Additional studies are required to define the signaling cascades initiated by these new NTFs and their physiological relevance to aging dopaminergic neurons.

**NTFs in pre-clinical and clinical studies of PD**

**Pre-clinical studies**

A commonly used approach to damage the NSS experimentally is to use dopaminergic toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA). This leads to an acute and severe neurodegeneration that is associated with behavioral deficits [25]. The use of NTFs for pharmacological purposes is limited by the fact that they do not cross the blood–brain barrier (BBB), have reduced diffusion rates and are rapidly degraded. NTFs must thus be delivered directly into the brain, at sufficiently high levels to bypass diffusion and stability constraints. GDNF and several other NTFs were found to exert neuroprotective, neurorestorative and stimulatory effects on toxin-treated SN neurons (Table 1) (reviewed in [26–30]). The newly identified CDNF/MANF are at least as potent as GDNF in providing neuroprotection and repairing rat SN neurons exposed to 6-OHDA [21,31]. Interestingly, MANF was transported to the cortex after striatal injection, in contrast to GDNF and CDNF, which were transported to the SN [31]. MANF might therefore exert its functions via a mechanism that is different from that used by GDNF.

Unlike dopaminergic-specific toxins, the bacterial endotoxin lipopolysaccharide (LPS) causes dopaminergic neurodegeneration via activation of surrounding glial (mainly microglial) cells [32]. Glial cell activation (neuroinflammation) is believed to be a contributing factor to PD [33] and GDNF protects dopaminergic neurons cultured in the presence of glia against LPS toxicity, by preventing microglia activation [34].

Although the mechanisms underlying the effects of MANF/CDNF on intoxicated neurons are unknown, those responsible for the effects of GDNF are beginning to be elucidated. GDNF protects against MPTP toxicity in mice and, interestingly, requires transforming growth factor-β (TGF-β) to exert its protective effect [35]. Ret-deficient SN neurons are not more sensitive to MPTP toxicity in vivo, but their axons fail to resprout during recovery [36]. Mice carrying a constitutively active RetMEN2B receptor also exhibit the same sensitivity to MPTP as wild-type mice. Thus, endogenous Ret signaling is neither required nor sufficient to protect SN neurons from MPTP toxicity, but mediates neurorestoration of MPTP-damaged axons. GDNF might therefore use its alternative receptor (NCAM) to protect against MPTP toxicity. Interestingly, when 6-OHDA was used to damage the NSS, RetMEN2B mice displayed increased protection of SN cell bodies, but not of dopaminergic terminals [37], indicating that the
requirements for neuroprotection might depend on the toxins used to challenge the NSS. The downstream signaling mechanisms that mediate the protective effects of GDNF remain elusive. Indirect evidence comes from viral-mediated delivery of constitutively active Akt to mice, which increased SN neuron size and sprouting during aging and exerted both protective and restorative effects when adult animals were challenged with 6-OHDA [38]. Akt signaling is thus mediating part of the neuroprotective and restorative effects of GDNF on diseased neurons.

A major drawback of these studies is the incapacity of toxin models to mimic PD pathology closely. Whereas degeneration in PD has an adult onset and is progressive, toxin-induced degeneration is acute and severe [1]. Therefore, the mechanisms behind NTF action in PD patients might be different from those in toxin-induced models. Great effort is currently being directed toward the development of improved animal models of PD. Animals that carry the same mutations as those found in familial PD might represent one solution, but such animal models generally fail to exhibit overt SN degeneration despite displaying several pathological alterations that parallel the human disease [1]. For example, combined deletion of all three PD recessive genes (those encoding DJ-1, Parkin and PTEN-induced putative kinase 1 (PINK1)) did not cause degeneration of aging SN neurons in mice, suggesting that these genes are dispensable for SN neuron survival in the mouse [39]. Does reduced NTF support contribute to PD? The experimental evidence that NTF disturbances alone cause PD is currently weak. A recent study [40] identified one patient with PD carrying compound heterozygous mutations in Ret and FK506 binding protein 4 (FKBP52) genes. Combined action of GDNF and nerve growth factor (NGF) is required to phosphorylate tyrosine 905 of the long isoform of human Ret (Ret51), which facilitates the interaction between FKBP52 and Ret51; the mutations identified in this patient impaired the formation of the Ret51–FKBP52 complex, without affecting Ret51 or FKBP52 expression levels. Mutations in GFRα2 (the co-receptor for NRTN that also binds GDNF) were recently associated with tardive dyskinesia, a late-onset movement disorder [41]. Further studies will determine the relevance of endogenous NTFs to the onset and progression of PD.

Although decreased NTF function might not be sufficient to cause PD, genetic inactivation of NTF signaling in animals carrying PD-associated mutations could be used to investigate the interplay between NTF signaling and PD-associated proteins. A recent study found that the combined deletion of DJ-1 and Ret caused adult-onset and progressive degeneration of SN neurons in mice, which exceeded that seen with Ret inactivation alone. Interestingly, the degeneration was specific to SN neurons that express the G protein-coupled inwardly rectifying potassium channel (GIRK2) [42], which might be more vulnerable in PD than the neighboring calbindin-expressing SN neurons [43]. Thus, the survival-promoting activity of PD-associated genes might be revealed in a background of impaired NTF signaling. However, mice lacking DJ-1 and Ret displayed no accumulation of α-syn and were not significantly impaired in motor tasks [42], suggesting that NTF manipulation can be used to probe a subset of pathogenic events in nigral disease. Complementary investigations in Drosophila revealed that DJ-1 interacts genetically with Ret-associated Ras/ERK signaling [42]. Thus, mounting evidence suggests that the cellular processes controlled by PD-associated genes are connected to NTF signaling (Box 1; [44]). Collectively, these studies raise several possibilities. First, PD-associated genes require an intact NTF network to promote SN neuron survival during aging. Second, the pathophysiological changes induced by mutations in PD-associated genes decrease the efficacy of NTF signaling. Third, the shared substrates between NTFs and PD-associated proteins might represent new targets for drug development in PD.

Clinical studies

Four clinical trials in which GDNF was administered to PD patients have been conducted so far (Table 2) (reviewed in [27,28,45–47]). Injection of GDNF into the cerebrospinal fluid (CSF) led to no significant improvement, probably because of its poor penetration from the CSF into the brain parenchyma [48]. To achieve better penetration, GDNF was directly infused (using minipumps) into the putamen in subsequent trials. The second and third open label trials found a significant clinical improvement [49–51], whereas the fourth, double-blinded placebo-controlled trial detected no improvements [52]. Some patients from trial 4 developed antisera against GDNF, although no adverse side effects were detected [52]. In a parallel study, monkeys receiving high doses of GDNF (three to four times higher than those used in human trials) developed cerebellar lesions [53]. For these reasons, the company that licensed the use of recombinant GDNF (Amgen) took the decision to stop clinical work on GDNF. Several possible explanations for the different outcomes in these trials have been proposed, including differences in GDNF doses or catheter properties, the choice of patient cohorts or the selection of inappropriate endpoints. A major limiting factor in the abovementioned trials was suggested to be the suboptimal brain delivery of GDNF [27,45,46].

Maximizing the potential of NTFs in PD

The experimental and clinical evidence obtained so far with NTFs suggests that sustained and localized NTF delivery is crucial for producing beneficial results. To achieve this, several strategies for improved NTF delivery are currently being developed, including the intracranial delivery of viral vectors, naked and/or encapsulated cells or microspheres and the intravenous injection of NTFs fused to antibodies that cross the BBB. In addition, NTF signaling might also be activated using small-molecule agonists (Figure 2).

Viral vectors

The most promising approach for NTF delivery in PD patients is gene therapy using recombinant adeno-associated viruses (AAVs) or lentiviruses (LVs) to infect neurons [28,30,47]. Pre-clinical studies found that AAV–GDNF or LV–GDNF delivery is sustained and leads to robust GDNF synthesis in the host brain and, in contrast to the previous clinical trials using recombinant GDNF, such delivery
Box 1. Convergence points between NTF signaling and PD-associated proteins

As detailed below, multiple putative interaction points between NTF signaling and PD-associated proteins have been identified (Figure I).

- **Ras/ERK.** DJ-1 synergizes with Ras and c-Myc to transform HEK293T cells [94] and cooperates with Ras/ERK signaling in *Drosophila* [42]. LRRK2 protects cells from H2O2-induced apoptosis by activating ERK signaling [95].

- **PI3K/Akt.** DJ-1 controls PTEN and Akt activity in hypoxic [96] or stressed [97] cells. At nanomolar concentrations, α-syn might be protective by stimulating PI3K/Akt signaling [98]. Parkin promotes PI3K/Akt signaling by preventing the degradation of the epidermal growth factor receptor [99] and de-represses NF-κB signaling by inhibiting the negative regulators tumor necrosis factor receptor-associated factor 2 (TRAF2) and inhibitor of kappa B kinase gamma (IKKγ) [100].

- **c-Jun N-terminal kinase (JNK).** DJ-1 inhibits the pro-apoptotic JNK signaling by preventing the activation of apoptosis signal-regulating kinase 1 (ASK1) by Daxx [101] or by preventing the nuclear transport of MEK kinase 1 (MEKK1) [102]. Parkin attenuates JNK and caspase-3 activation to protect against 6-OHDA [44] or rotenone [103] toxicity.

- **p53.** DJ-1 inhibits p53 transcriptional activity by directly binding to p53 [104,105] or by interacting with the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex [106]. Inhibition of p53 by DJ-1 seems to be Akt dependent, but ERK and NFkB independent [105]. Parkin interacts with the p53 promoter and inhibits p53 transcription, in an ubiquitin–ligase-independent manner [107].

- **Caspases.** Mutant LRRK2 interacts with Fas-associated death domain protein (FADD) to induce caspase-8-mediated cell death in cultured neurons [108]. Caspase-8-mediated cleavage of DJ-1 generates a C-terminal fragment that translocates to the nucleus to inhibit p53 and caspase-3 activity [109]. Mutant LRRK2 causes increased caspase-3 activation, which depends on the presence of LRR and WD40 domains of LRRK2 [109].

- **Mammalian target of rapamycin (mTOR).** Activation of mTOR by PI3K/Akt inhibits autophagy and autophagy stimulation (using the mTOR inhibitor rapamycin) prevented dopaminergic neurodegeneration in Parkin or PINK1 mutant flies [110]. LRRK2 directly phosphorylates and inactivates the eukaryotic translation initiation factor 4E-binding protein (4E-BP), which inhibits translation [111]. Consequently, deletion of *Drosophila* LRRK2 promoted autophagy by enhancing 4E-BP activation and suppressed neurodegeneration in Parkin and PINK1 mutants [110].

- **Mitochondria.** Parkin has autonomous effects on mitochondrial mechanisms regulating cytochrome c release in neuroblastoma cell lines [112]. PINK1 modulates the activity of the mitochondrial protease HtrA2 by promoting p38-dependent HtrA2 phosphorylation [113].

- **Calcium.** PINK1 controls mitochondrial Ca2+ efflux by regulating the Na+-Ca2+ exchanger, thereby preventing mitochondrial overload with Ca2+ and oxidative stress [114]. Mutant PINK1 or α-syn increase mitochondrial Ca2+ influx and cause defects in neurite outgrowth, which could be rescued by blocking the mitochondrial Ca2+ uptake channel [115]. However, most of these biochemical interactions await confirmation in SN neurons in vivo.

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**Figure I.** The intracellular signaling pathways activated following NTF binding to an NTF receptor (NTF-R) might share common targets with PD-associated proteins.
Table 2. Clinical trials with neurotrophic factors and neurotrophic factor inducers in PD

<table>
<thead>
<tr>
<th>Drug class</th>
<th>NTF</th>
<th>NTF inducer</th>
<th>MAO-B I/NTF inducer</th>
<th>NTF inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance</td>
<td>GDNF</td>
<td>GDNF</td>
<td>AAV2-NRTN</td>
<td>Rasagiline</td>
</tr>
<tr>
<td>Trial type</td>
<td>MRDBPC</td>
<td>OL</td>
<td>MDBSSC</td>
<td>DBPCDST</td>
</tr>
<tr>
<td>Trial phase</td>
<td>I/II</td>
<td>I</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td>Duration (months)</td>
<td>6-8</td>
<td>12-24</td>
<td>12-18</td>
<td>12</td>
</tr>
<tr>
<td>Delivery mode</td>
<td>ICV</td>
<td>IPu</td>
<td>IPu</td>
<td>IPu</td>
</tr>
<tr>
<td>Daily dose</td>
<td>14-43.2 mg</td>
<td>3-30 mg</td>
<td>15 mg</td>
<td>N/A</td>
</tr>
<tr>
<td>Number of participants</td>
<td>50</td>
<td>5</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58 ± 8</td>
<td>54.2 ± 6</td>
<td>57.9 ± 9.3</td>
<td>56 ± 7.2</td>
</tr>
<tr>
<td>Time since diagnosis (years)</td>
<td>11 ± 6</td>
<td>19 ± 9.8</td>
<td>8.7 ± 3.6</td>
<td>9.7 ± 3.9</td>
</tr>
<tr>
<td>Clinical benefits?</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Side effects</td>
<td>LS and WL</td>
<td>LS</td>
<td>LS</td>
<td>H</td>
</tr>
<tr>
<td>Trial ID</td>
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<td>NCT00006488</td>
<td>NCT0015427</td>
<td>NCT00252850</td>
</tr>
<tr>
<td>Refs.</td>
<td>[48]</td>
<td>[49,50]</td>
<td>[51]</td>
<td>[52]</td>
</tr>
</tbody>
</table>

Abbreviations: H, headaches; ICV, intracerebroventricular delivery; IPu, intraputaminal delivery; LS, Lhermitte’s sign; MAO-B I, monoamine oxidase type B inhibitor; MDBSSC, multi-center, double-blind, sham-surgery controlled trial; MRDBPC, multi-center, randomized, double-blind, placebo-controlled trial; N/A, not applicable or not available; NS, not significant; RDBPC, randomized, double-blind, placebo-controlled trial; Tu, tumors; WL, weight loss.

*Recombinant human methionyl GDNF (r-methHuGNDF).

This MRDBPC trial was followed by an OL on 16 PD patients who received up to 4-mg GDNF for an additional 20 months; no improvements were observed.

*Drug injected (6-8 times; in total 25-4000-mg GDNF).

*Delivered via minipump.

*Recombinant virus was injected once (dose: 1.3 × 10^11 or 5.4 × 10^11 viral genomes).

*Clinical improvement was assessed using the Unified Parkinson’s Disease Rating Scale (UPDRS); note that other functional and non-motor criteria have been used in some of these studies.

*A modest improvement of AAV2-NRTN treatment was observed after 18 months in a subset of participants that had double-blind assessments after 12 months.

*Only the most prevalent side effects are listed; for a complete list, see cited references.

*LS represents a sudden but transient electric shock that extends down the spine and is triggered by flexing the head forward.

*Three AAV2-treated patients and two controls receiving sham surgery developed tumors; tumors in the AAV2-NRTN group are thought to be unrelated to the presence of AAV2-NRTN.


*A new clinical trial with recombinant GDNF is expected to begin soon [65].

*A new trial, in which a higher (fourfold) dose of AAV2-NRTN will be delivered to both the putamen and the SN is underway [65].

*This trial was continued as an OL for an additional 5.5 years, and the clinical benefits of early rasagiline treatment were maintained [85].
**Figure 2.** Ways to activate NTF signaling in the nigrostriatal system: the example of GDNF. (a) Direct infusion of recombinant GDNF into the brain parenchyma or the cerebrospinal fluid (not shown) is facilitated by the use of minipumps. (b) Cells engineered ex vivo to produce GDNF continuously can be implanted as such (naked cells) or encapsulated into catheter-like devices made of polymers, which are permeable to nutrients and enable outward diffusion of newly produced GDNF (encapsulated cells). (c) Small molecules that target diverse aspects of GDNF signaling can be used to enhance GDNF signaling. These molecules (or their metabolites) must be able to pass the BBB. Molecules that resemble GDNF (green) or that bind and activate Ret (yellow) or GFRα1 (orange) can mimic GDNF action. Molecules that antagonize negative regulators of GDNF–Ret signaling, such as Lrig1 (black) or Sprouty (grey), or which antagonize (blue) or activate (pink) inhibitors or activators of Ret-downstream effectors (e.g., Akt) might also mimic activation of GDNF–Ret signaling. Small compounds, such as rasagiline or cogane™, stimulate NTF (including GDNF) production. Given the potential risk for side effects associated with widespread activation of GDNF signaling, small compounds that target GDNF signaling specifically in dopaminergic neurons might be more advantageous. (d) The use of molecular ‘Trojan horses’ might facilitate the entry of NTFs into the brain. For example, the N-terminus of GDNF can be fused to the C-terminus of the heavy chain of a monoclonal antibody directed against the human insulin receptor (Mab HIR) to generate a chimeric protein (GDNF-Mab HIR). Following intravenous injection, the chimera binds the HIR, which facilitates its transport across the BBB. Once in the brain, the N-terminus of GDNF recruits GFRα1 and activates Ret signaling. A major caveat of this approach is the widespread delivery of GDNF throughout the brain, which might cause side effects. (e) Viral vectors enable the in vivo delivery of GDNF by introducing the gene that encodes it into striatal neurons. Lentiviruses (LVs) carry the GDNF cDNA surrounded by viral long terminal repeats (LTRs), packaging signal (cPPT), the rev responsive element (RRE) required for nuclear export of RNA, the central polypurine tract (cPPT) that enhances the nuclear import of dsDNA, a promoter, and a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) that increases the nuclear export of GDNF mRNA. Adeno-associated viruses (AAVs) contain inverted terminal repeats (ITR), the GDNF transgene and a promoter. Both LVs and AAVs are produced in cell lines by co-transfection of plasmids containing the GDNF transgene and packaging plasmids. (f) Microspheres that bind or entrap GDNF can be implanted into the putamen, where they are degraded and GDNF is released. Given that microspheres can pass the BBB, they can also be injected intravenously (not shown).
phase I human trial, one year post-administration [63] (Table 2). However, phase II testing on an extended cohort failed to show significant improvements [64] and post-mortem evidence indicated that NRTN showed poor diffusion into the tissue [64,65]. While a new trial with AAV2–NRTN is underway (cited in [65]), three other recombinant AAVs (encoding metabolic enzymes that act to enhance dopaminergic neuron function or which normalize the activity of the basal ganglia) are currently being tested in phase I/II trials [2,47].

**Naked or encapsulated cells**

Cells engineered *ex vivo* to secrete NTFs represent another delivery strategy. Human neural progenitor cells producing GDNF transplanted into the rat striatum populated most of the target area and mitigated 6-OHDA-induced neurodegeneration [66]. Bone marrow stem cell-derived macrophages are able to cross the BBB and LV-transduced macrophages producing GDNF protected SN neurons from MPTP toxicity in rats [67]. Another strategy is to use encapsulated cell biodelivery (ECB) that, in contrast to naked cells, uses cells encapsulated in catheter-like devices made of polymers; these devices ensure a local NTF delivery and can be removed surgically if desired [68]. ECB of GDNF alleviated SN neurodegeneration in 6-OHDA-treated rats [69]. However, it only provided transient motor improvements in MPTP-treated monkeys, probably because the intraventricular grafting did not enable sufficient GDNF to diffuse from the CSF into the brain [70].

**Microspheres**

Submicroscopic spherical assemblies consisting of natural or synthetic polymer aggregates can be used to attach or entrap NTFs. These nanoparticles can cross the BBB, are biodegradable and enable the slow release of NTF into the desired area. GDNF released from polymeric lactide–glycolide acid (PLGA) microspheres implanted in the striatum was neuroprotective [71] and neurorestorative [72] in 6-OHDA-treated rats. Similarly, lactoferrin-bound GDNF, given intraperitoneally or intravenously, protected dopaminergic neurons from 6-OHDA [73] or rotenone [74] toxicity in rats. Further pre-clinical studies are required to assess the safety and efficacy of this peripheral delivery approach.

**‘Trojan horse’-mediated delivery**

Another approach for NTF delivery into the CNS is the intravenous administration of a chimeric protein comprising the NTF and a so-called ‘molecular Trojan horse’, for example a monoclonal antibody (Mab) to the human insulin receptor (HIR) that facilitates the receptor-mediated passage of the chimera across the BBB. Rhesus monkeys injected intravenously with a GDNF–MabHIR chimera displayed increased brain uptake of GDNF, without side effects, 2 weeks after administration [75]; a phase I safety trial in humans is now underway [65]. A major drawback of these peripheral delivery approaches is the widespread delivery of GDNF throughout the CNS, which might cause unwanted side effects. A possible solution for this has recently been tested in rats via the use of Trojan horse liposomes to deliver a plasmid in which the GDNF gene is placed downstream of a dopaminergic-specific promoter [76].

**NTF agonists**

Another strategy to activate NTF signaling is to use small-molecule NTF agonists. DNSP-11 is a small peptide from the pro-GDNF domain that stimulates and restores SN neurons after 6-OHDA damage *in vivo* [77]. Interestingly, it does not bind GFRα1 but interacts with several metabolic proteins and might thus exhibit neurotrophic activities through GFRα1-independent pathways [77]. The small nonpeptidyl compound XIB4035 is a weak agonist for GFRα1 and stimulates Ret autophosphorylation and neurite outgrowth in neuroblastoma cells [78], although this compound has yet to be tested *in vivo*. A high-affinity TrkB agonist, 7,8-dihydroxyflavone, stimulates TrkB autophosphorylation and signaling *in vivo* and protects SN neurons in MPTP-treated mice [79]. Several natural compounds and pharmacological agents were found to increase NTF production [80]. Rasagiline is one such compound that, although functioning as a monoamine oxidase B (MAO-B) inhibitor, also upregulates GDNF and brain-derived neurotrophic factor (BDNF) production [81] and is neuroprotective in MPTP-treated mice and monkeys [82]. In two delayed-start clinical trials, rasagiline showed positive results [83–85] (Table 2), which might reflect potential disease-modifying effects [86,87]. Another orally active compound is PYM50028 (Cogane™), which increases GDNF and BDNF expression and exhibits neuroprotective and neurorestorative effects in MPTP-treated mice [88]; this compound is now in Phase I clinical testing (Table 2). Finally, new compounds that activate NTF signaling specifically in dopaminergic neurons (or are targeted to these neurons specifically) might prove useful for therapy and the identification of dopaminergic-specific NTF interactors might facilitate the screening for such compounds.

**Conclusions and perspectives**

Pre-clinical studies have uncovered a promising potential for NTFs to repair the damaged NSS system and to reverse motor deficits in parkinsonian animal models. The lack of definitive therapeutic effects in human trials should not be seen as complete failure, but rather as an opportunity to improve theoretical and practical approaches to NTF therapeutic strategies (Box 2). One of the most pressing questions is to understand how NTFs impact on aging and diseased dopaminergic neurons. It is possible that the aging human brain has a decreased capacity to respond to NTFs, which is further impaired by disease. In addition, the effects of NTFs on other neuronal populations should be characterized. Given the plethora of NTFs and their complex roles in the brain, it remains possible that these factors can be used to repair neuronal populations outside of the NSS that become affected in PD and which are responsible for the non-motor manifestations of PD. This knowledge could also be used to maximize the therapeutic effect and to minimize side effects following NTF delivery. The ongoing investigations into the basic biology of NTFs and their behavior in pre-clinical experiments will unravel the complex molecular interplay between NTFs...
Box 2. Outstanding questions

- How do NTFs interact with the cellular machinery that regulates aging? The aging brain might have a reduced capacity to respond to NTF stimulation, and this might limit the efficacy of NTF therapies for PD. Several mechanisms that regulate aging in model organisms have been identified [89], and it would be interesting to determine whether and how NTF action is altered by these factors.
- What are the effects of NTFs on non-dopaminergic neurons? Which of these effects might be deleterious when administering NTFs to patients with PD? Alternatively, these effects might have positive therapeutic benefits for treating the non-motor symptoms of PD.
- Can genetic manipulation of NTF signaling pathways in animal models mimic aspects of PD? Is it possible to model the action of NTFs on dysfunctional dopaminergic neurons? The use of large-scale approaches (i.e. proteomics and transcriptomics) might facilitate the in silico analysis of dopaminergic neuron responses to injury and to NTFs, and might lead to the identification of new therapeutic targets that modulate the NTF system.
- Is reduced neurotrophic support a cause of PD?
- Are the current criteria for clinical evaluation of PD (e.g. the Unified Parkinson’s disease rating scale [UPDRS]) sensitive enough to enable the detection of disease-modifying effects? Or are more sensitive clinical indicators needed?
- How early can PD be detected? Although an accurate diagnosis of PD is currently difficult, most cases already display >60–70% loss of SN neurons and >80% loss of dopamine before being clinically detected [25]. Under these conditions, there is little room for neuroprotective effects. Therefore, achieving neuroprotection with NTFs urgently requires validated biomarkers to detect pre-symptomatic stages of PD.
- Is a combination of several NTFs more efficient in protecting, repairing and activating dopaminergic neurons in the diseased brain compared with a single NTF? GDNF requires TGF-β to exert its neuroprotective effects [35]; thus TGF-β might be valuable clinically for enhancing the effects of GDNF.
- How can the diffusion of GDNF or NRTN into the brain parenchyma be improved? These two factors have reduced diffusion capacity, which might contribute to their failure in clinical trials. Mutant proteins that have reduced affinity for heparan sulfate proteoglycans (HSPGs) could be generated and tested in vivo; however, caution should be exerted as complete disruption of HSPG binding might abolish neuroprotection [116]. The new NTFs (CDNF and MANF) have better diffusion capacities [31] and might be good alternatives for future trials.
- Is long-term expression of NTFs using LVs or AAVs safe in humans, or should a regulatory system that can shut off transgene expression be used? Although AAVs appear to be safe in ongoing clinical trials [63], and no safety concerns arose from the pre-clinical use of LVs [30], future generations of viral vectors that are engineered with the possibility to turn off gene expression, might be highly desirable. One approach that has been tested experimentally is the use of dietary doxycycline, which was used to turn off the expression of a GDNF transgene that was downstream of a reverse tetracycline promoter in AAV–GDNF-infected rats [117].

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