Opinion

Can Growth Factors Cure Parkinson's Disease?

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Growth factors (GFs) hold considerable promise for disease modification in neurodegenerative disorders because they can protect and restore degenerating neurons and also enhance their functional activity. However, extensive efforts applied to utilize their therapeutic potential in humans have achieved limited success so far. Multiple clinical trials with GFs were performed in Parkinson's disease (PD) patients, in whom diagnostic symptoms of the disease are caused by advanced degeneration of nigrostriatal dopamine neurons (DNs), but the results of these trials are controversial. This review discusses recent developments in the field of therapeutic use of GFs, problems and obstacles related to this use, suggests the ways to overcome these issues, and alternative approaches that can be used to utilize the potential of GFs in PD management.

Neurotrophic Factors and Their Potential in Parkinson's Disease Therapy

Neurodegeneration in Parkinson's disease (PD; Box 1) starts from the axons of dopamine (DA) neurons (DNs) in the putamen (Figure 1). When PD is diagnosed there is a 70–80% reduction in the putaminal DA level, approximately 70% decrease in the density of DA axons, but only 30% reduction in the number of DN cell bodies in the substantia nigra pars compacta (SNpc) [1,2]. Therefore, the remaining functional and dystrophic DNs represent an attractive target for supportive and **neurorestorative** (see Glossary) therapeutic approaches, respectively, which can be provided by growth factors (GFs) including neurotrophic factors (NTFs; Box 2).

GFs have been extensively tested in several preclinical PD models (PD models are described in Box 3) and have shown considerable promise for disease modification. Some of them were also trialed in PD patients, but the therapeutic success of such interventions was rather limited. To critically evaluate the results of clinical trials conducted with GFs in PD patients it is important to understand key differences in pharmacology and mode of action of GF treatments compared with conventional small-molecule drugs. Importantly, GFs act in the organism via defined and physiologically relevant molecular pathways in contrast to other agents which are potentially **neuroprotective** for DN (e.g., an antidiabetic drug exenatide) [3]. Moreover, GFs not only protect, but also restore neurons, promote arborization and sprouting of their neurites, and enhance the functional activity of neurons. These points justify further attempts to translate GFs into drugs for PD treatment.

Special Aspects of GF Functioning Relevant for Their Clinical Translation

Biological activity of endogenous GFs in mammals is tightly controlled. GFs usually have a short half-life *in vivo*. In the brain they are often released in a pulsatile manner and are rapidly bound to their receptors in target tissues, internalized, recycled, or degraded. The short-term interaction of GF with its receptor is sufficient for activation of intracellular signaling cascades necessary for neuronal survival and functioning. Then the second messenger proteins in signaling cascades activate transcription factors that trigger the expression of their target genes. While the

Highlights

Growth factors (GFs) supporting neuronal survival and increasing their functional activity are promising leads for the development of disease-modifying treatments for neurodegenerative disorders.

Clinical use of GFs requires careful planning with regard to dosing, delivery paradigms and equipment, frequency of administration, and the development of patients' stratification criteria.

Several alternatives to naturally occurring GFs, such as mutant proteins or small molecules targeting GF receptors, show promise in animal models of Parkinson's disease (PD) and can progress to clinical use with better outcomes.

Early diagnostics of PD and immediate initiation of the treatment may significantly improve clinical perspectives of GF-based therapeutics.

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Box 1. Parkinson's Disease: Prevalence, Symptoms, Pathophysiology

Parkinson's disease (PD) affects 0.3% of the population, being more frequent in males and the elderly. The usual onset of the disease occurs at an age of 65–70 years [73]. In the brains of PD patients dopamine (DA) neurons in substantia nigra pars compacta (SNpc) degenerate and eventually die. This produces characteristic motor symptoms of the disease such as tremor, rigidity, and slowness of movement, which form the basis for PD diagnosis. In addition to motor symptoms, PD patients experience a number of non-motor symptoms including gastrointestinal tract disturbances, anosmia, sleep disturbances, depression, sexual function deterioration, neuropsychiatric problems, postural hypotension, and cognitive decline associated with the degeneration or dysfunction of other neuronal populations in the brain and other organs. Non-motor symptoms may precede motor symptoms by several years or even decades [74,75].

Histopathological examination reveals the presence of Lewy bodies in the brains of PD patients. Lewy bodies are bulky protein aggregates predominantly consisting of α-synuclein, but also more than 90 other proteins [76]. Prevailing concept considers Lewy bodies as a cause of neurodegeneration by, for example, impairing axonal transport, although some authors indicate their potential neuroprotective role mediated by sequestering cytotoxic oligomers of α-synuclein [76]. Importantly, there is an immense need in finding biomarkers for early and reliable diagnostics of PD. Currently the diagnosis is based on manifestation of motor symptoms which appear when extensive degeneration in the nigrostriatal DA system has already occurred, and even in the presence of motor symptoms it can be difficult to diagnose PD at the early stage of the disease as some of the motor symptoms may also appear in patients with other conditions. Introducing new diagnostic methods based, for example, on the analysis of non-motor symptoms in combination with positron emission tomography (PET) scanning using dopamine transporter (DAT) tracers can greatly improve the efficacy of neurorestorative treatments allowing therapeutic intervention to be initiated when the lesion is mild and significant number of cells can be protected and healed. Noteworthy, however, that non-motor symptoms are not specific for PD and patients with other diseases often mention them as well. Finally, neurorestorative treatments should be initiated immediately after diagnosis as the disease is progressive and the remaining DNs gradually continue to degenerate, leaving only a small number of cells that can be restored even in the 3–5 years after the diagnosis [5,6].

transmission of a signal in a signaling cascade is a rapid process occurring within several minutes after the application of a GF, transcriptional effects may result in significant rearrangements in cellular processes and last for many days or even months after the inactivation of the GF (Figure 2A).

By contrast, prolonged activation of GF receptors can be detrimental for their biological effects because of multiple negative feedback mechanisms in signaling cascades aimed to restrict uncontrolled propagation and multiplication of a signal [4]. GFs often produce biphasic dose–response curves in biological experiments [5] and in clinical trials [6], mediated by different mechanisms. For instance, high concentrations of GFs may inhibit the formation of functional oligomeric signaling complexes because each monomer of the receptor is bound to a ligand molecule [7]. GFs at high concentration can also trigger internalization pathways in the cell that elicit GF and receptor degradation in short-term (Figure 2B) or suppression of receptor's expression via various silencing mechanisms in long-term experiments [8]. Both of these processes can be further augmented by an insufficient amount of available chaperones necessary for efficient folding of newly synthesized GF receptors. Excessive amount of GF can also trigger negative feedback mechanisms in its downstream signaling cascades including dephosphorylation of activated receptors [9] or second messengers by phosphatases and block the transmission of signal.

Elevated levels of GFs caused by continuous overexpression or infusion may also cause more general negative effects in the DA system. In particular, glial cell line-derived neurotrophic factor (GDNF) overexpression was shown to downregulate the level of **tyrosine hydroxylase (TH)**, the key enzyme of DA synthesis (DA synthesis and signaling are shown in Figure 1D) in rats [10]. It can also cause aberrant sprouting and ectopic formation of synapses in the brain, thus producing multiple adverse effects that can be dose dependent [11].

Importantly, GFs are often expressed in several isoforms and the activity or even their biological effects can be different [12]. GFs are prone to degradation by intracellular and extracellular matrix proteases and their half-life and stability can depend on post-translational modifications.

Glossary

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): toxin that is widely used to model PD as it selectively destroys DA cells.

6-Hydroxydopamine (6-OHDA): toxin is widely used to model PD because it selectively destroys DA cells. 6-OHDA is given with an inhibitor of serotonin and noradrenalin reuptake, as it is also toxic to serotonergic and adrenergic neurons.

α-Synuclein: a protein involved in neurotransmitter release that is expressed in neuronal cells, especially in DNs at high level. α-Synuclein tends to aggregate especially when mutated and/or overexpressed. Aggregated α-synuclein is a major component of Lewy bodies detected in the brains of the majority of PD patients. According to general belief α-synuclein aggregates cause the neurodegeneration of DNs by interfering with cellular processes. However, there are data contrasting this view. Therefore, the final conclusion of significance and role of α-synuclein aggregation in PD is yet to be made.
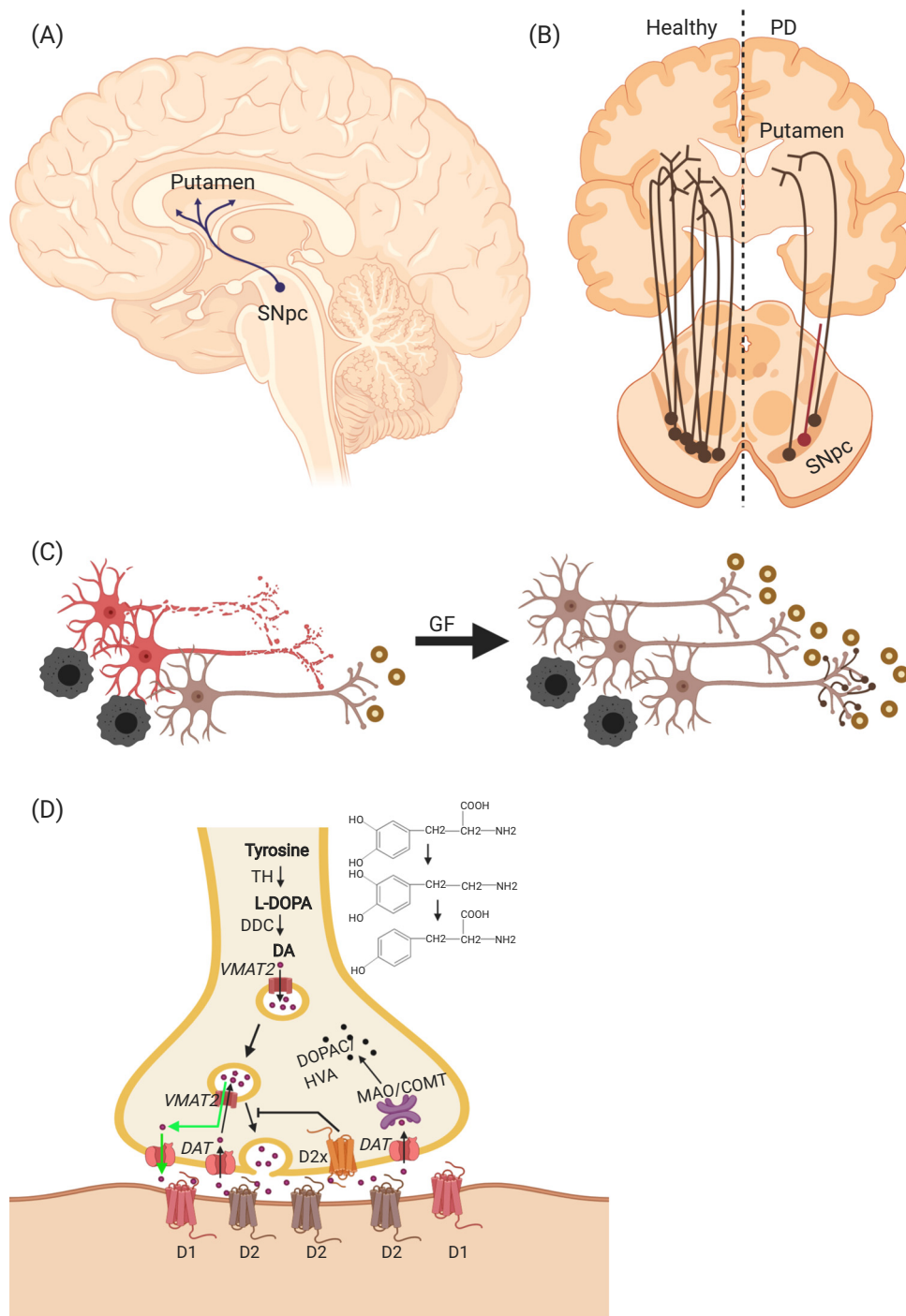
Dopamine transporter (DAT): a transmembrane protein responsible for reuptake of DA from the synaptic cleft into cytosol. It is a widely used marker of DNs in preclinical and clinical samples. The DAT is generally considered to express at a rather stable level through a variety of conditions and treatments.

Neuroprotective: an ability of the test substance to prevent the death of neurons when given before/simultaneously with a lesion.

Neurorestorative: properties that manifest as an ability of the test substance to structurally restore damaged cells by, for example, stimulation of the neurite outgrowth if given when the lesion is already established.

Positron emission tomography (PET): a method to estimate the level and distribution of radioactively labeled compound (tracer) in the living organism. In PD patients ^{18}F-DOPA is often used as a tracer to evaluate activity of DATs which to a certain extent correlates with the severity of nigrostriatal DA system degradation and motor impairment.

Tyrosine hydroxylase (TH): a key enzyme of DA synthesis that is widely used as a marker of DNs in preclinical and clinical samples. The level of TH in the cell can be upregulated and downregulated upon certain treatments.

(A)

Putamen

SNpc

(B)

Healthy | PD

Putamen

SNpc

(C)

GF

(D)

Tyrosine
TH
L-DOPA
DDC
DA
VMAT2

DOPAC
HVA
MAO/COMT

VMAT2

DAT

D2x   DAT

D1   D2   D2   D2   D1

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Figure 1. Nigrostriatal Dopamine (DA) System in Healthy and Parkinson's Brain (PD) and GF Actions in DA Neurons (DNs). (A) Nigrostriatal pathway on the sagittal section of the brain. (B) Condition of the DA system in healthy and PD brains, where DNs degenerate and eventually die (coronal sections). (C) Sites of actions of GFs in DNs. GFs do not resurrect dead DNs (dark gray cells), but they protect healthy cells (brown), restore injured cells (red), increase the functional activity of neurons (as shown by the high number of released mediator-containing vesicles, brown circles), and

(Figure legend continued at the bottom of the next page.)

Box 2. Neurotrophic Factors, Their Functions, and Signaling

Neurotrophic factors (NTFs) are small, secreted proteins mainly promoting the survival, protection, and restoration of injured neurons. Most NTFs are synthesized as preproproteins and then are proteolytically cleaved to release the mature form. Importantly, some proneurotrophic factors can also have biological effects different from those of mature NTFs. In particular, while nerve growth factor (NGF) elicits trophic support to neurons, pro-NGF stimulates apoptosis [77].

Many proteins can influence different aspects of neuronal functioning, but only those that are able to promote the survival of specific neuronal populations are considered NTFs. Currently, four families of NTFs have been discovered: neurotrophins, neurokines, GDNF family ligands (GFLs), and the cerebral dopamine neurotrophic factor (CDNF)/mesencephalic astrocyte-derived neurotrophic factor (MANF) family.

Proteins from the first three families bind to specific cell surface receptors with kinase activity and activate intracellular enzyme and kinase cascades such as PI3K/AKT, MAPK/ERK, Stat, Src, PLC-gamma, which are important for the survival and functioning of neuronal cells (see Figure 2 in the main text) [78]. The receptors transmitting neuroprotective signals from neurotrophins are tropomyosin receptor tyrosine kinases A, B, and C (Trk A–C). Neurotrophins and proneurotrophins can also signal via the p75 neurotrophin receptor (p75^{NTR}), a transmembrane glycoprotein and a member of the tumor necrosis family (TNF) of receptors. Neurokine signaling occurs via a complex of ligand-selective low-affinity receptor and signaling transmembrane gp130 receptors, which are constitutively associated with JAK (Janus kinase)-family tyrosine kinases. GFLs signal mainly through the receptor tyrosine kinase rearranged in transfection (RET) and glycosylphosphatidylinositol (GPI)-anchored coreceptor GFRα1–4 which provides ligand binding specificity. CDNF/MANF proteins are localized in the endoplasmic reticulum and signal inside cells, but can be also secreted. Extracellularly delivered CDNF and MANF protect and restore DA neurons in animals models of PD [21,60,79,80], thus implying the existence of signal-transducing extracellular receptors or internalization mechanisms for these proteins. The structure of MANF and CDNF suggests that they can interact with lipids [81] which can in turn mediate intracellular uptake of these proteins as was shown for lipid sulfatide instead of traditional transmembrane protein receptors [82]. *In vitro* and preclinical studies clearly demonstrate neurorestorative properties of GFLs and CDNF/MANF in the nigrostriatal DA system, while neurotrophins mainly have neuroprotective effects. Neurokines might have some effects in proliferation of DN precursor cells, which seems to take place in mice, but occurs with very low frequency in humans (reviewed in [78]). Therefore, in this review, we mainly focused on effects of GFLs and MANF/CDNF proteins in PD.

Biological activity and stability of recombinant GFs produced in bacterial systems which are used in clinical trials can also differ from those of protein produced in eukaryotic cells [13,14].

The majority of clinical trials in PD patients were conducted using continuous infusion of high doses of recombinant GFs or their constitutive overexpression from viral vectors in the brain because GFs generally fail to penetrate through the blood–brain barrier (BBB) and often poorly spread in tissues [15]. Therefore, they have to be either injected directly into the brain in the vicinity of target neurons by means of complicated stereotactic surgery, modified to improve their BBB penetration, or delivered using special techniques, such as nanoparticles. Problematic delivery, features of biological responses of GFs, and their specific pharmacological properties limited the success of clinical trials and resulted in premature conclusion on the lack of GF efficacy in PD.

GFs in Animal Models of PD: Effects and Problems in Clinical Translation

Many GFs show the ability to protect DNs in animal models of PD (PD models are described in Box 3). Several, in particular GDNF, neurturin (NRTN), mesencephalic astrocyte-derived neurotrophic factor (MANF)/cerebral dopamine neurotrophic factor (CDNF), and platelet-derived growth factor (PDGF), also showed neurorestorative effects in experimental animals. Detailed description of GF effects in

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may induce axonal spouting. (D) Synapse diagram of a DN. DA is synthesized from amino acid tyrosine via two enzymatic reactions catalyzed by tyrosine hydroxylase (TH; rate-limiting step) and DOPA decarboxylase (DDC) and packed into secretory vesicles with the help of vesicular monoamine transporter 2 (VMAT2). Released DA in putamen binds to postsynaptic DA receptors type 1 and 2 (D1–2) and interacts with presynaptic DA receptor D2x mediating negative regulation of DA release. DA is reuptaken back into the presynaptic neuron terminal via the DA transporter (DAT) where it can be either degraded by monoamine oxidase/catechol-O-methyltransferase (MAO/COMT) or repacked into secretory vesicles by VMAT2. DOPAC/HVA (3,4-dihydroxyphenylacetic acid/homovanillic acid), or DA metabolites. Green arrows represent the pathway which is stimulated by DA-releasing agents, such as amphetamine. Abbreviations: L-DOPA, dihydroxyphenylalanine; SNpc, substantia nigra pars compacta.

Box 3. Modeling PD in Experimental Animals: Emerging Methods

To model PD in experimental animals toxins, mechanical lesions, genetic manipulations, and α-synuclein preformed fibrils are all used [83,84]. None of available models represents all PD features on a mechanism-related basis. Measurable parameters in animal models of PD are motor deficits and non-motor symptoms. Also used is the status of the DA system by functional (e.g., DA release or electric activity) and morphometric analysis of the nigrostriatal system (immunohistochemically using antibodies against DN markers, e.g., TH, the key enzyme for DA synthesis), the dopamine transporter (DAT), the vesicular monoamine transporter 2 (VMAT2; Figure 1D) or neuronal dyes such as Cresyl violet (Nissl staining).

The models of toxin-induced PD are well validated and widely used in drug discovery, but shed little light on disease mechanisms. Multiple attempts have been made to develop better models of PD by knocking-in or knocking-out PD-related genes in experimental animals. A vast majority of genetic models show mild and poorly reproducible DN degeneration although motor impairment and functional deficits are often reported. Perhaps the main limitation of most genetic PD models is related to the fact that only 5–10% of patients have familial PD developed as result of inheritance of mutated genes. Therefore, such models are representative only for minority of disease mechanisms [85]. Genetic models based on overexpression or mutations in α-synuclein gene are clearly different in this regard as α-synuclein aggregates are found in the brains of the majority of PD patients. Overexpression of α-synuclein from viral vectors was reported to induce the loss of DNs, produce motor symptoms, and stimulate protein aggregation in nigrostriatal pathway [86], but these data turned out to be difficult to reproduce and could have resulted from high levels of exogenous protein in the brain [27,84]. Transgenic animals expressing wild-type or linked to human PD mutant α-synuclein generally exhibited only mild, if any, degeneration of DNs [87]. Recently a new transgenic mice line characterized by the age-dependent loss of DNs caused by expression of truncated 1–120 α-synuclein fragment has been established [88]. Also, initiation of Lewy body-like pathology accompanied by the loss of TH-positive cells in SNpc was shown to be stimulated by preformed α-synuclein fibrils injected into rodent brain [89,90]. A MitoPark model of PD was developed by selective deletion of mitochondrial transcription factor Tfam in DNs and is characterized by slow progressive decline in motor function and striatal DA content, thus resembling the mitochondrial mechanism-based disease pathology as mitochondrial degeneration is associated with PD development [91]. These three models may be very useful in the late stage of anti-PD drug discovery.

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animal models is reviewed elsewhere [16–18]. Here we will discuss several important issues related to clinical translation of preclinical findings.

First, although GFs show profound effects in partial neurotoxin lesion models, their effects are much less robust or even absent in animal models of severe PD [19–21]. At the same time, clinical studies with GFs are mainly conducted in the late-stage PD patients who have lost the majority of striatal DNs fibers and have much less nigral DNs compared with early stage patients, which complicates the detection of potential therapeutic effects, especially in functional outcomes [1,22].

Another issue concerns the influence of protein aggregation on the expression of GF receptors. Several publications reported that overexpression of **α-synuclein** from viral vectors in rodent brain results in downregulation in expression of many genes, including GDNF receptor RET (Figure 2B) and its upstream regulator, the transcription factor Nurr-1 [23,24]. Based mainly on these data, one may conclude that limited efficacy of GDNF observed in clinical trials is related to α-synuclein-induced decrease in RET expression. However, the expression of α-synuclein mRNA in the brains of PD patients is not upregulated [25], instead it can even be downregulated [26]. Evidence showing downregulation of GF receptors and Nurr-1 in the brains of PD patients are absent [25]. Moreover, mild increase in α-synuclein mRNA level in rodent brains also fails to downregulate the expression of NTF receptors [25]. It is also unclear whether the decrease in RET and Nurr-1 expression [23,24] is caused specifically by α-synuclein aggregation or just appears as a result of high level of heterologous protein in the brain in animal α-synuclein overexpression models of PD [27].

DNs in humans and experimental animals also have several important differences. At first, human brain is much bigger compared with rodent brain. Every human midbrain DN forms 1–2 million synapses, while rat neurons produce only 100 000–250 000 [28]. This results in a huge difference in the energy and intracellular trafficking demand in these neurons. In addition, human neurons should stay alive and function for much longer time compared with rodents' because of

(A)

(B)
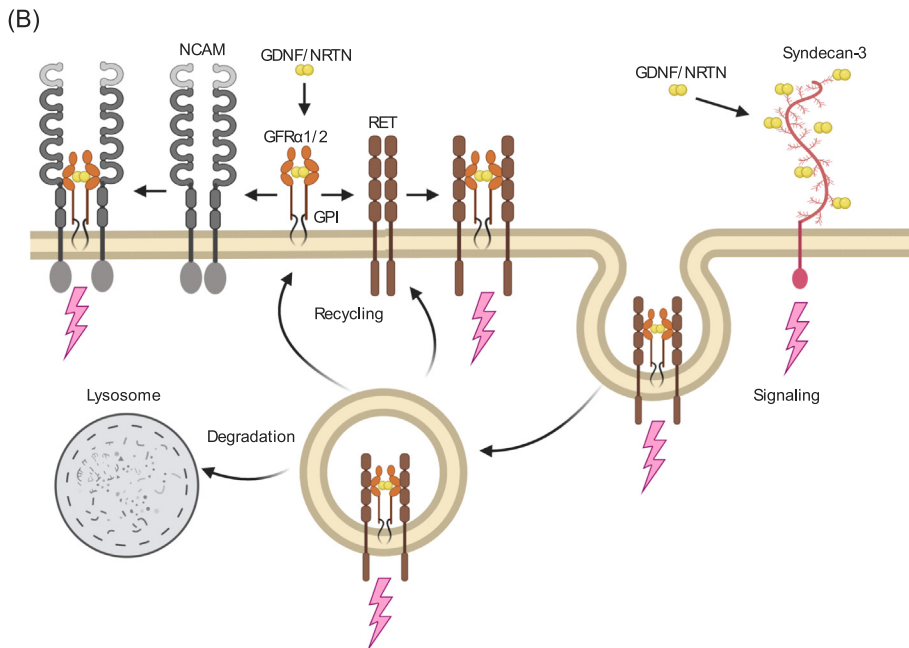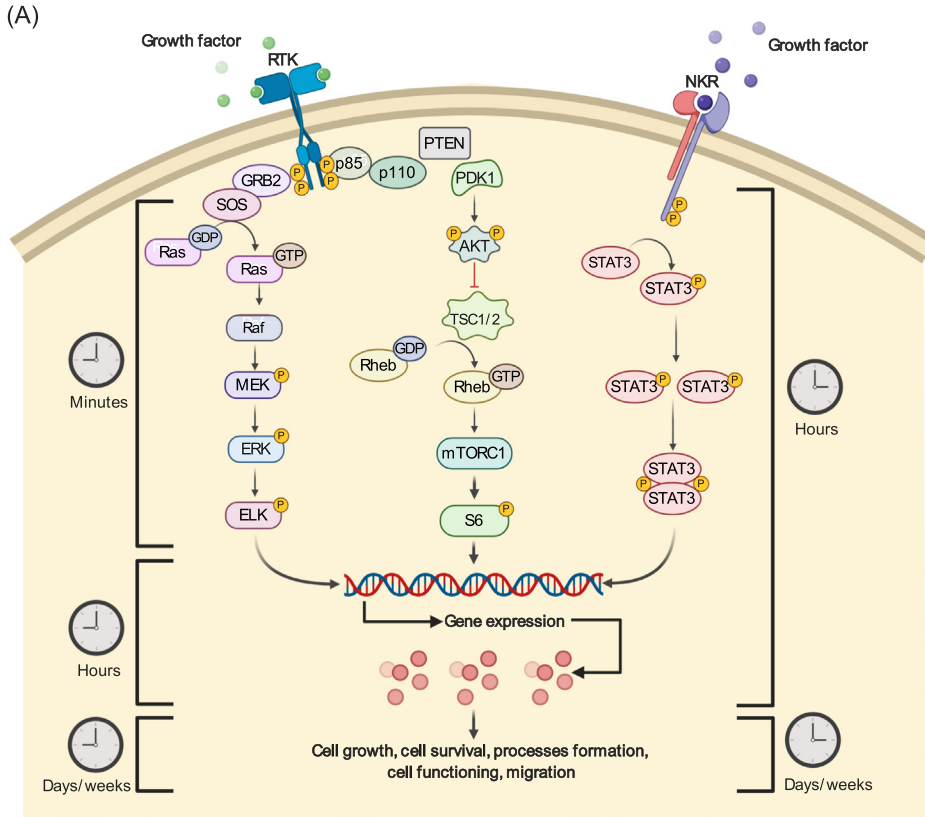
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interspecies difference in the life span. Although the exact reasons for DN vulnerability and death in PD are unknown, some studies suggest that their normal activity can be a risk factor because of neurotoxic effects of DA [29] and/or dysfunction of calcium homeostasis [30]. Thus, rodent DNs may exhibit different susceptibility to degenerative and regenerative stimuli compared with human cells. Recent achievements in stem cell research may finally result in the development of animal models with grafted human stem cell-derived DNs for drug testing, thus improving translation perspectives of novel PD therapeutics.

GFs can also have important applications in stem cell-based therapies that now are being trialed in PD patients (reviewed in [31]), as they promote survival and functional integration of the grafted cells [32].

GFs in Clinical Trials in PD Patients

Based on promising results in moderate neurotoxin-lesioned animal models of PD, four GFs (PDGF, GDNF, NRTN, and CDNF) were chosen for clinical trials in PD patients. Intracranially delivered recombinant PDGF protein has been, and CDNF protein is being, tested in small-scale Phase I/II clinical trials in PD patients with the main goal to evaluate the safety and tolerability of the treatment as well as signs of efficacy. PDGF was well tolerated and its main adverse effects were associated with implantation of the delivery device. It improved the **dopamine transporter (DAT) function** in **positron emission tomography (PET)** scans at least in some patients. All patients showed improvement in motor function evaluated using the **Unified Parkinson's Disease Rating Scale (UPDRS)** by an average of 4.5 points compared with baseline, but no differences between placebo and PDGF-treated groups were reported [33]. In animal models of PD, PDGF was shown to stimulate neurogenesis in the subventricular zone and thus improve the condition of the nigrostriatal DA system [34]. While reported in some model organisms, it is unclear if neurogenesis occurs in adult human midbrain [35]; therefore, the lack of PDGF efficacy in PD patients may be related to mechanistic difference between species.

A 1-year CDNF Phase I/II clinical trial was completed in August 2020; however, the main results are yet to be reported. The data from the first 6-month evaluation show that CDNF is safe for patients and improves DAT function in PET imaging in some patients, thus supporting the disease-modifying potential of this protein in PD (Herantis Pharma Plc and University of Helsinki, press releases[i,ii]). According to the newsletter published by Herantis Pharma Plc, the treatment was found to be safe also after 12 months of administration and showed signs of efficacy such as motor and DAT function improvements at least in some patients[iii].

GDNF family ligands (GFLs) have been studied in PD patients much more extensively. In the first Phase II clinical trial, intraventricularly delivered GDNF did not improve motor performance of

Figure 2. Growth Factor (GF) Signaling. (A) Simplified scheme of GF signaling in the cells, showing, as an example, activation of mitogen-activated protein kinase (MAPK)/protein kinase B (AKT) pathways via receptor tyrosine kinase (RTK) and signal transducer and activator of transcription (STAT) pathway via the neurokine receptor (NKR). GFs bind to cell surface receptors and via transmembrane receptor cytoplasmic domains activate intracellular second messengers. This leads to stimulation of gene expression and results in cellular events, such as survival and neurite outgrowth. RTKs mediate both rapid events via changes in phosphorylation status of multiple proteins and long-lasting effects via activation of transcriptional mechanisms. NKRs mainly activate transcription of various proteins in the cells. (B) Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) signaling. Homodimeric GDNF and NRTN bind to glycosylphosphatidylinositol (GPI)-anchored coreceptors GFRα1/2 and activate a transmembrane receptor tyrosine kinase (RET). The signaling complex is internalized and either degraded or recycled. Intracellular signaling cascades are activated by both cell surface and endosomal receptor/ligand complex. In the presence of GFRα1 receptor GDNF can also signal via neural cell adhesion molecule (NCAM) or can directly bind to and signal via Syndecan-3. Abbreviations: ERK, extracellular signal-regulated kinase; GFRα1/2, GDNF family receptor alpha-1/2; GRB2, growth factor receptor-bound protein 2; MEK, mitogen-activated protein kinase kinase; mTORC1, mammalian target of rapamycin complex 1; PDK1, 3-phosphoinositide-dependent protein kinase-1; PTEN, phosphatase and tensin homolog.

patients as it failed to reach target neurons due to its inability to cross cell barriers. Serious treatment-related adverse effects such as nausea, anorexia, weight loss, paresthesia, and pain were reported [36]. Better understanding of GFLs biodistribution features resulted in two small-scale Phase I open-label trials in which intraputaminally delivered GDNF decreased UPDRS values and increased fluorine-18-dihydroxyphenylalanine (^{18}F-DOPA) uptake on PET scans by 19% in the absence of significant adverse effects [37,38]. The effect of the treatment persisted for several months after the withdrawal of treatment [39]. A subsequent Phase II randomized placebo-controlled clinical trial designed to replicate these findings failed to reach its primary endpoints [40]. Although the treatment was rather well tolerated, some adverse effects (mainly paraesthesia and headache) and the development of GDNF-neutralizing antibodies in some patients were reported. The design of this clinical trial, GDNF dose, GDNF delivery system, and administration regimen have received significant criticism [41–43]. Formation of anti-GDNF antibodies (which remained largely unexplained and can only be associated with peripheral leakage of GDNF) together with cerebellar toxicity of high doses of GDNF reported in monkeys [44] transformed the delivery protocol in GDNF clinical trials to a more physiological pulsatile one. Intermittent convection-enhanced administration of GDNF produced neither toxic effects in non-human primates [45] nor anti-GDNF antibodies in PD patients [46]. The results of a recent Phase II clinical trial indicated that intermittent intraputaminal administration of GDNF is well tolerated. Although this study also failed to reach its primary endpoints, all patients in the GDNF-treated group had significantly higher ^{18}F-DOPA uptake on PET scans; in *post hoc* analysis 43% of them showed an improvement in UPDRS by at least 10 points (compared with 0% in the placebo-treated group) and, importantly, 95% of patients receiving GDNF for 80 weeks had clinically significant improvements in at least one outcome measure [46,47]. Similarly, adeno-associated virus 2 vector (AAV2)-encoded GDNF produced an increase in ^{18}F-DOPA uptake in patients and was shown to be safe and well tolerated [48].

Another GFL, NRTN, was administered to PD patients with the help of AAV2 vector (AAV2-NRTN, and CERE-120). Similar to GDNF, intracranially delivered AAV2-NRTN was safe for patients in both short- and long-term studies [49–51]. At first, AAV2-NRTN was infused only into putamen, but postmortem analysis of the brains of several patients revealed the presence of NRTN protein only in a few DN bodies in SNpc, suggestive of impaired retrograde transport [52,53]. Therefore, in the latest clinical trial CERE-120 was infused into both striatum and SNpc. Neither of the clinical trials with AAV2-NRTN met primary endpoints, meaning that the patients showed no improvements in functional outcome measures.

Important to note here is that the assessment of outcomes of clinical trials in PD patients is complicated by placebo-related responses which are reported in the majority of these trials. Analysis of the data of 858 PD patients assigned to placebo groups in 11 clinical trials revealed that the overall placebo response rate (defined as ≥50% improvement in UPDRS score or a decrease by ≥2 points on 2 or more UPDRS items) was in average equal to 16% [54]. In another small study (n = 12) the magnitude of placebo-induced response in acute settings showed up to 28% reduction in UPDRS score [55].

GDNF [56] or CERE-120 [57] increased the density of TH-positive fibers in putamina of PD patients. In patients treated with CERE-120, TH expression comparable in intensity with that seen in the brains of healthy age-matched controls was detected in areas also positive for NRTN [53,57]. In addition, the levels of pERK (phosphorylated extracellular signal-regulated kinase) and pS6, second messengers in mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) signaling cascades (Figure 2A) triggered by NRTN via RET, were elevated in SNpc of patients treated with CERE-120 delivered into both putamen and SNpc [57]. These data show that GFLs are also able to produce trophic effects in human DNs.

However, overall increase in the density of TH-positive fibers in putamina of CERE-120-treated patients was rather modest and highly variable. The portion of putamen with increased TH staining did not exceed 30%; the density of remaining TH-positive fibers was highly variable and constituted only 3.9–21.6% of that in age-matched controls [57].

GFLs have high affinity for extracellular matrix and cell surface heparin sulfates containing proteins (Figure 2B) and, therefore, they poorly diffuse in tissues [15]. Thus, only a portion of putamen is covered by infused or viral vector-encoded GFL. The portion of putamen covered by GDNF in the clinical trial conducted by Lang *et al.* [40] was estimated to be in the range of 2–9% [43]; in Whone *et al.*'s [46,47] clinical trial, up to 57%; and in Heiss *et al.*'s trial [48], on average about 26%; in the AAV2-NRTN trial, NRTN expression was detected in postmortem brain samples in 3.75–22% of putamen [53,57]. These estimates were made by immunochemistry [43,57] or based on the distribution of the contrasting agent gadolinium [46–48]. It is possible that neither of these methods fully reflects the distribution of GFs in effective concentrations in the brain. The effective concentrations of GFs may be lower than that detected by antibody or an antibody can detect very low concentrations of GFs insufficient to produce a biological response. In this regard, the analysis of GF-induced signaling in DNs can be a better method and it is possible that the putamen coverage by GFLs in patients is overestimated.

One of the major limitations of clinical trials with GFs in PD is the need for surgical delivery of the drug directly into the brain. This leads to the selection of patients with moderate to advanced PD in clinical trials because of ethical constraints. Indeed, all clinical trials with GDNF and NRTN were conducted in patients with PD duration of at least 10 years in average. However, *post hoc* analysis of AAV2-NRTN clinical trial data revealed that the most responsive cohort is patients with a duration of PD below 5 years [58]. This can be explained by the progressive nature of PD and low number of DNs and their fibers which GFs are expected to support and restore in late-stage patients [1]. It was shown that only a few TH- and DAT-positive fibers remained in the putamina of patients 5 years after PD diagnosis and overall reduction in optical density of TH-positive fibers at this time point reached up to 90%, remaining at the same level for a decade or even two [22]. Even in early stage PD patients (3 years postdiagnosis) the degree of DA fibers loss varied from moderate to marked [22]. Thus, the variability produced by differences in GFL diffusion, extent of trophic effects, stage of the disease, and the rate of disease progression make it difficult to see statistically significant motor improvement in patients when all of them are pooled together. The development of better spreading variants of GFs and patient stratification in clinical trials are necessary future steps in clinical translation of GFs in PD.

Potential Alternatives to Native GDNF and NRTN in Translational Research

Obstacles related to the delivery and tissue distribution of wild-type GFs have stimulated research focused on finding alternative methods (summarized in Table 1, Key Table) to utilize their clinical potential in PD. Modified GFs have been developed and tested in animal models of PD. Mutagenesis in heparin-binding domains of GDNF and NRTN produced several variants with greatly improved diffusion in brain parenchyma [14,59]. GDNF mutants had reduced functional activity [59], but NRTN variants exhibited significantly enhanced activity *in vivo* [14].

MANF and CDNF proteins diffuse in the striatum better compared with GFLs [21,60]. Additionally, structurally they include two biologically active domains and can, therefore, be split to further reduce the size of protein and improve their biodistribution [61]. Full-length MANF and CDNF, however, still have to be delivered intracranially, as they fail to cross through the BBB; thus their clinical use can be limited by ethical restrictions in patient selection which

Key Table

Table 1. Problems Related to Clinical Use of GFs and the Ways to Resolve Them

Problem	Possible solution(s)
Requirement for surgical delivery due to inability to cross BBB	Development of fusion proteins, peptides, or small molecules penetrating through BBB
Poor spreading in target tissues	Using convection-enhanced delivery system, alternative GFs with better tissue distribution, such as CDNF, mutant GFs, peptides, or small molecules
Biphasic dose–response curves	Intermittent delivery of close to physiological doses of GFs, regulated overexpression from viral vectors, administration of short-lived small molecules or peptides at regular intervals (e.g., once in 3 days)
Variability of response to the treatment in the subpopulations of patients	Patient stratification criteria in clinical trials should be developed. Early stage patients seem to respond to the treatment with GFs better. Rate of disease progress may also play a role.
Treatment-associated adverse effects	Intermittently delivered GFs at close to physiological doses seem to be well tolerated by patients. Continuous infusion or constitutive high-level overexpression may have detrimental effects and have to be avoided.
Development of antibodies to GFs	Development of anti-GF antibodies is unlikely if GFs are delivered into the brain. The integrity of containers and tubing implanted in patients have to be closely monitored. Small molecules and peptides can be used as non-immunogenic alternatives.
Surgery-related complications in clinical trials	BBB-penetrating forms of mutant GFs, small molecules, and peptides can be used.
Variations in biological activity between batches of recombinant proteins	Biological activity rather than quantity of GF should be assessed for each batch. Production of GF in mammalian cells may result in more physiological post-translationally modified (e.g., glycosylated) forms and improve biological activity and stability.
Detrimental effects of constitutive overexpression or injection of high doses of recombinant GF with regard to their biological activity (receptor downregulation, negative feedback mechanism activation, gene expression downregulation, etc.)	Intermittent delivery of proteins and regulated expression of viral vectors with aim to deliver close to physiological doses of GFs may represent a way to overcome these negative effects and progress with clinical trials in PD patients. The safety of intermittent delivery paradigm was shown in the latest trials with GDNF protein [46,47]

favor the choice of late-stage patients due to the invasiveness of the treatment and brain surgery-related complications.

To eliminate the need for intracranial delivery, GDNF was fused with peptides binding to transport proteins in BBB, such as HIV-1-Tat-derived cell-penetrated peptide (Tat-GDNF) [62] and heavy chain of monoclonal antibody against transferrin receptor (TfRMAb-GDNF) [63]. Intraperitoneally injected Tat-GDNF crossed the BBB and reached nigrostriatal DNs, but showed no neuroprotective activity in **1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)**-treated mice, perhaps because of either insufficient concentration in the brain or the lack of functional activity (which was not assessed in the study) [62]. Functionally active TfRMAb-GDNF penetrated BBB [although the brain uptake was rather low (about 3% of intravenously injected dose)] [63], was well tolerated [64], improved motor function, and increased TH activity in striata of mice in the **6-hydroxydopamine (6-OHDA)** model of PD [65]. Chronic administration of TfRMAb-GDNF led to the development of antibodies

against TfRMAb, but not GDNF itself, which seemed to have no influence on pharmacokinetics of the fusion protein [64].

In rodents intranasal delivery of either naked or nanoparticle-protected plasmids encoding GDNF was shown to protect DNs from 6-OHDA-induced death [66]. Whether this approach can also stimulate neurorestoration and if it is applicable to humans is yet to be studied, as previous attempts to use gene therapy in PD showed significant differences between model organisms and patients in terms of, for example, transport of virally encoded protein from striatum to SNpc [52].

Another option is to target the receptors of GFs with peptides or small molecules able to penetrate through BBB and spread in the body targeting motor and non-motor symptoms of PD via trophic support to both DN and, for example, olfactory, basal forebrain, enteric, and other neurons.

GDNF-derived peptides targeting one of its receptors neural cell adhesion molecule (NCAM) supported the survival of cerebellar granule cells and promoted neurite outgrowth from both these and DNs *in vitro*. However, they failed to support the survival of DNs in culture [67] which indicates that NCAM is unlikely to play a significant role in this process. A peptide containing 11 sequential amino acid residues from the proregion of GDNF (DNSP-11) increased the survival of cultured DNs. DNSP-11 delivered into SNpc was internalized by DNs in healthy rats, reduced motor symptoms, and increased the concentration of striatal DA in the rat 6-OHDA PD model with a marked lesion. However, it signaled via different receptors compared with GDNF as it failed to bind GDNF family receptor alpha-1 (GFRα1), retained activity in the presence of the kinase inhibitor staurosporine, and in a pull-down assay interacted mainly with metabolic proteins which are not downstream targets of the GDNF/GFRα1/RET axis [20].

To the best of our knowledge no biologically active peptides selectively targeting the main GFL receptor RET have been described so far and our own attempts to design such molecules have remained unsuccessful (Y.A. Sidorova *et al.*, unpublished). We, nevertheless, developed three structurally unrelated chemical scaffolds selectively targeting and activating RET [68–71]. We showed that compounds belonging to one of these scaffolds penetrate through the BBB, support the survival of cultured DNs, protect them from MPTP- and 6-OHDA-induced cell death, stimulate release of DA in mouse brain, and alleviate motor symptoms in the 6-OHDA model of PD [68,72]. The second and third scaffolds are yet to be tested in the DA system. Further optimization of these chemicals to improve their biological activity and druglike properties may eventually convert them into orally administered disease-modifying treatments against PD.

Concluding Remarks

Although attempts to use GFs in clinical settings have a long history, these proteins are yet to demonstrate their full potential in PD patients. GF-based therapeutics are different from conventional small-molecule drugs in many aspects and their use requires careful design of clinical trials (see Outstanding Questions). Patient stratification, dose, and the delivery method should be meticulously considered in the future studies of GFs in PD patients. Nonlinear dose–response and pulsatile nature of GF release under physiological conditions favors intermittent administration of moderate doses of the proteins or the use of regulated gene therapy vectors in clinical trials in order to limit upregulation of various negative feedback mechanisms. Patient recruitment strategy should favor the selection of early stage PD patients to ensure the participation of people with sufficient numbers of remaining DNs in SNpc. Clinically favorable alternatives such as mutated GFs with improved tissue distributions, small molecules targeting GF receptors, and BBB-penetrating peptides should be seriously considered for further development of disease-modifying treatments against PD. Using alternatives to GF with improved tissue-penetrating ability,

Outstanding Questions

What are the best doses for GFs in clinical trials in PD patients?

How can we limit the detrimental effects of high doses of GFs in regard to downregulation of gene expression, reduction in the number of receptors on cell surface, and activation of negative feedback mechanisms in the cells?

How can we select the most responsive PD patients for GF-based treatments?

What is the best way to target GF receptors in PD patients?

How can we improve tissue spreading of GFs?

Is there a possibility to avoid the need for brain surgery which is currently used to deliver GFs into the brains of PD patients?

Can we utilize the potential of GFs to target non-motor symptoms in PD patients?

Is it possible to target both motor and non-motor symptoms of PD with one drug?

for example, small molecules, can also provide a way to target not only motor, but also non-motor symptoms of PD. In summary, clinical trials with GF-based therapeutics in PD patients should be continued in the future to find a cure for millions of affected people, but their design should be considerably improved.

Disclaimer Statement

M.S. is a co-founder and shareholder of Herantis Pharma, a company which conducts clinical development of CDNF. Y.A.S. and M.S. are shareholders of GeneCode Ltd, a company owning the patent for one scaffold of RET agonists.

Resources

[i]https://herantis.com/press_releases/herantis-pharma-plc-announces-topline-results-of-phase-1-2-cdnf-trial/

[ii]https://www.helsinki.fi/en/news/health-news/initial-clinical-data-shows-promising-signals-of-the-biological-activity-of-cdnf-in-parkinsons-disease

[iii]https://herantis.com/wp-content/uploads/2020/09/Newsletter_Sep_2020_ENG.pdf

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