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REVIEW

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Theoretical Approaches to Lentiviral Mediated Neurotrophin Delivery in Potential Treatments of Parkinson's Disease

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Parkinson's disease is a late-onset neurodegenerative disease, characterized by both motor and non-motor symptoms. Motor symptoms include postural instability, rigidity, and tremor, while non-motor symptoms include anxiety, dementia, and depression. In this integrative review, we discuss PD disease pathophysiology in detail and introduce how neurotrophic growth factor delivery via a retroviral-based system can be used as efficacious tools for targeted gene therapy.

INTRODUCTION

Parkinson's disease (PD†) is a late-onset neurodegenerative disease, characterized by both motor and non-motor symptoms. Motor symptoms include freezing, postural instability, rigidity, and tremor, while non-motor symptoms include, anxiety, dementia, and depression [1]. Pathologically, it involves the loss of dopaminergic neurons in the nigrostriatal pathway (located in the midbrain) and the widespread accumulation of Lewy bodies (intracellular aggregates of the alpha-synuclein protein) in the central and peripheral nervous systems [2] that cause local inflammation. Furthermore, many areas of the midbrain also experience a drastic depletion of the neurotransmitter dopamine [3].

Currently, an array of potential treatments exist, attempting to target the above mechanisms. Prevailing therapeutics include small molecule inhibitors targeting gene expression of both the leucine-rich repeat kinase (*LRRK2*) gene as well as the alpha-synuclein (*SNCA*) gene [4], protein delivery mechanisms and gene therapy approaches to deliver neurotrophic factors like neurturin (*NRTN*) and glial cell line-derived neurotrophic factor (*GDNF*) [5,6], transplantation of totipotent stem cells in adult brains and anti-inflammatory drugs like coenzyme Q, minocycline, and caspase (which induces apoptosis) along with pathway inhibitors, all aimed to provide neuroprotective effects [5,7,8]. Although some of the proposed techniques temporarily alleviate symptoms, a non-invasive, target-specific treatment that will lead to long-term remission is ideal. Furthermore, some of the proposed treatments have not yet been implemented in clinical use.

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†Abbreviations: PD, Parkinson's disease; *LRRK2*, leucine-rich repeat kinase 2; *SNCA*, alpha-synuclein; *NRTN*, neurturin; *GDNF*, glial cell line-derived neurotrophic factor; *PINK1*, PTEN-induced putative kinase 1; *PER1*, period circadian clock 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; CNS, central nervous system; *NGF*, nerve/neural growth factor; *BDNF*, brain-derived neurotrophic factor; NT-3, neurotrophin-3; TNFα, tumour necrosis factor alpha; BBB, blood brain barrier; PEP-1-HO-1, PEP-1-hemeoxygenase-1; *ROS*, reactive oxygen species; GF, growth factor; *NTR*, neurotrophin; *EGF*, epidermal growth factor; LGF, liver growth factor; IGF-1, insulin-like growth factor 1; TGFα, intrastriatal transforming growth factor alpha; *FGF20*, fibroblast growth factor 20; AAV2, adeno-associated virus type 2; 6-OHDA, 6-hydroxydopamine; HIV, human immunodeficiency virus; SCID-X1, X-linked severe combined immunodeficiency; VSV-G, vesicular stomatitis virus; hDAAC, human aromatic L-amino acid decarboxylase; FMT, fluoro-L-M-tyrosine; DA, dopamine; *GAD*, glutamic acid decarboxylase; UPDRS, unified PD rating scale; RRV, ross river virus; LCMV, lymphocytic choriomeningitis virus; RD114, feline endogenous retrovirus; *GFAP*, glial fibrillary acidic protein.

Keywords: Parkinson's disease, lentivirus, neurotrophin, microglia, alpha synuclein

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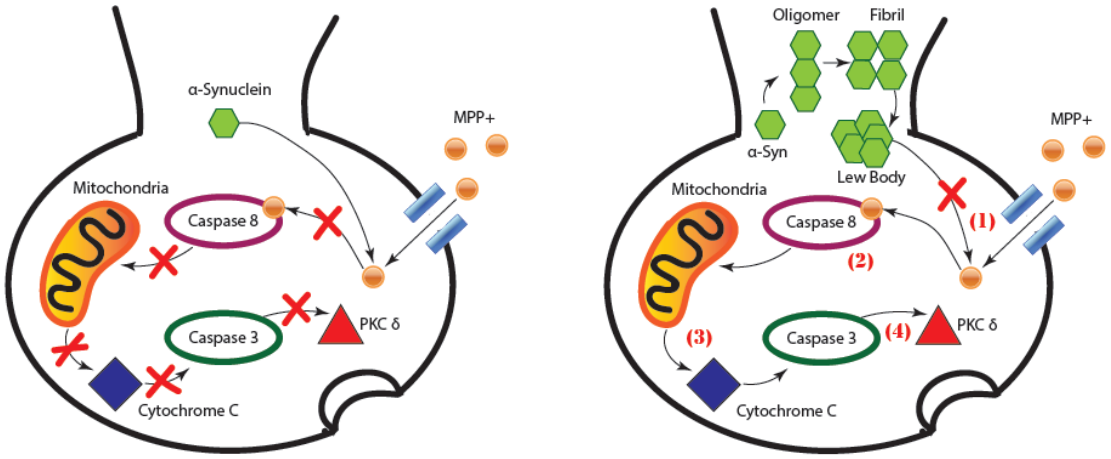

A.1 Wild Type Alpha-Synuclein

A.2 Mutated Alpha-Synuclein

Figure 1. The neuroprotective properties associated with wild type α-synuclein and the neurotoxic effects associated with mutated α-synuclein. A) The interaction between α-synuclein and MPP+ (1-methyl-4-phenylpyridinium) in the caspase 3-mediated apoptotic cascade. The binding of α-synuclein to MPP+ prevents the cascade from occurring and by extension, prevents cell death. B) The aggregation of α-synuclein into oligomers and fibrils to form Lewy bodies. (1) Lewy bodies are unable to bind to MPP+ and therefore the cascade proceeds. (2) MPP+ binds to the caspase 8 molecule to activate it. Caspase 8, with the MPP+ bound, approaches mitochondria. (3) Cytochrome c is released due to caspase 8 signaling and *ROS* (reactive oxygen species) are produced, contributing to cell death. Cytochrome c then activates caspase 3, the molecule that induces apoptosis. (4) Caspase 3 is responsible for the cleavage of PKCδ (a protein kinase) and subsequently triggers neuronal apoptotic cell death in mesencephalic dopaminergic neurons. Image Credit: Netra Unni, Faculty of Applied Science and Engineering, University of Toronto.

The following sections detail the fundamental components underlying the etiology of this multi-system neurodegenerative disorder.

Disease Pathophysiology

The pathophysiology has been linked to four distinct mechanisms: the formation of intraneuronal inclusions known as Lewy bodies (aggregates of the alpha-synuclein protein), genetic mutation in various genes such as *LRRK2*, *PINK1*, *SNCA*, PRKN and the development of chronic inflammation as a result of oxidative and proteolytic stress, eventually leading to the degeneration of dopaminergic neurons in the substantia nigra pars compacta [4,9,10].

Lewy Bodies

The widespread accumulation of Lewy bodies in the central and peripheral nervous systems is an essential neuropathological characteristic of PD progression. The major constituent of Lewy bodies is the protein α-synu-clein. Spillatini et al. (1998) utilized immunohistochemistry techniques to verify the presence of α-synuclein. The use of the primary antibody, *PER1* (an anti-α-synuclein antibody) and the secondary anti-ubiquitin antibody enabled complete and strong staining of Lewy bodies in the midbrain tissues of patients with PD. Their findings suggested that these protein aggregates contain full-length or close to full-length α-synuclein, forming a majority of the abnormal filaments that constitute Lewy bodies [11].

Located on chromosome 4, α-synuclein is encoded by the *SNCA* gene. A mutation in this gene has been associated with familial cases of PD. The protein is present in both water-soluble and lipid-based neurological tissues, allowing its existence in the intra-neuronal environment [12]. It is abundant in the synapse and believed to play a role during synaptic vesicle release [11]. The protein exists as a monomer and aggregates by forming oligomers and fibrils, subsequently [13]. A prominent hypothesis in the mechanism of neuronal cell death, induced by Lewy bodies, involves the caspase 3-mediated apoptotic cascade [14,15]. Figure 1 outlines possible pathways of alpha synuclein aggregation in a neuron containing wild-type α-synuclein and mutated α-synuclein.

Genetic Mutations

Although there are cases of both sporadic and familial PD, current research exemplifies increasing evidence of genetic mutations as a significant contributing factor to the pathogenesis of PD. Specifically, the two main target genes are *LRRK2* and *SNCA*.

The *LRRK2* gene codes for is a *leucine-rich repeat kinase* known as dardarin. The gene product also plays a role in many biological interactions including the retrograde trafficking pathway for recycling proteins, synaptic

Table 1. An overview of growth factors and their potential functions in PD.

Growth Factor	Potential function	References
Neurturin	Will reduce the degeneration of neurons and enable neurons to function more efficiently	Olanow et al., (2015), Bartus et al., (2013), Wang et al., (2012), Tanriover et al., (2010), Ye et al., (2007)
Neurotrophin	Will enable quick and efficient integration of nigral grafts to the native neuronal tissue	Haque et al., (1996), Tong et al., (2009), Nagatsu et al., (2000), Mogi et al., (1999) and Ebadi et al., (1998)
Epidermal Growth Factor	Will upregulate the expression of *EGF* receptors and by extension increase afferent signals of dopaminergic neurons	Iwakura et al., (2005), Chen et al., (2011) and Pellecchia et al., (2013)
Liver Growth Factor	Will promote the proliferation and tissue regeneration in the substantia nigra pars compacta and other regions of dopaminergic neuron degeneration	Gobernado et al., (2013) and Reimers et al., (2012)
Insulin-like Growth Factor 1	Will display neuroprotective effects and reduce cognitive impairment as a result of PD	Offen et al., (2001), Kim et al., (2012), Godau et al., (2010) and Mashayekhi et al., (2010)
Transforming Growth Factor	Will stimulate the proliferation, migration and differentiation of dopaminergic neurons in the striatum and substanatia nigra	Cooper, Isacson (2004) and Espejo et al., (2001)
Fibroblast Growth Factor 20	Will enhance the survival of dopaminergic neurons in the substantia nigra and will increase the levels of alpha-synuclein protein in the neurons	Zhu et al., (2015), Xu et al., (2013), Satake et al., (2007) and Mizuta et al., (2008)

vessicle release and protein phosphorylation, which has been postulated to play a central role in PD [3]. Berg et. al (2005) conducted a clinical study with 53 unrelated families and found that mutations of the *LRRK2* gene accounted for approximately 13 percent of apparently autosomal dominantly inherited PD. Thus far, a total of 10 missense mutations and one splice site mutation have been described with respect to the *LRRK2* gene.

With regards to the *SNCA* gene, researchers strongly believe that the mutations in this gene are responsible for the aggregation of α-synulcein and hence the formation of intraneuronal Lewy bodies. In autosomal dominantly inherited cases, it was observed that a pathogenic missense mutation in *SNCA* contributed to approximately 2.5 percent of cases, resulting in it being a rare causal PD gene [9].

Inflammation and Microglial Activation

Inflammation is observed as a result of oxidative or proteolytic stress, the activation of microglia, as well as the upregulation of cytokines in the midbrain and cerebrospinal fluid [16].

The pathways essentially follow a cause-effect loop. When dying neurons are faced with an imbalance in free-radical production, oxidative stress mounts in the cells. This stress leads to the activation of many transcription factors which in turn express genes either coding for or controlling the effect of inflammatory cytokines [17]. The inflammation further impacts the neuron's functioning and induces cell death [18].

Chronic inflammation can also be induced as a result of the body's natural response. The brain, particularly, is equipped with specialized immune cells known as microglia. Wu et al. (2002) discovered that the blocking of microglial activation by minocycline protects the nigrostriatal dopaminergic pathway that is characteristically targeted by parkinsonian toxins, such as 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This suggests that inflammation as a result of microglial activation plays a key role in the pathogenesis of PD [8].

The primary, innate immune cells found within the central nervous system (CNS) are the microglia (brain-specific macrophages) that are capable of exhibiting two distinct phenotypes: the pro-inflammatory (M1) type or the anti-inflammatory (M2) type [16]. Under normal physiological conditions, the microglial cells are ramified and make contact with neuronal axons, synapses as well as other glial cells such as astrocytes [19]. These interactions facilitate the secretion of neurotrophic factors such as nerve growth factor (*NGF*), brain-derived neurotrophic factor (*BDNF*), and neurotrophin-3 (NT-3) which promote neuronal growth and survival [20,21]. However, pathological conditions such as the formation of Lewy bodies in Parkinson's disease can activate the M1 phenotype of mi-

croglia [22]. Upon activation, the cells release pro-inflammatory chemical mediators, such as cytokines (e.g., interleukins, tumor necrosis factor (TNFα), chemokines (e.g., α-, β-, γ-, δ- classes), and humoral factors [23]. Accumulation of these factors sustain a pro-inflammatory response, intoxicating the surrounding neurons and compromising the integrity of the blood brain barrier (BBB) – a tightly regulated, selectively permeable membrane involved in ion homeostasis. Furthermore, they attract macrophage progenitor cells (e.g., monocytes) from the bone marrow to migrate to the CNS and differentiate into active microglia [24,25]. Microglial proliferation and chronic activation, in turn, contribute to disease progression and deterioration of the patient's quality of life.

Degeneration of Dopaminergic Neurons

Although PD also results in the degeneration of other neurons, including serotonergic and noradrenergic, it has been observed that degeneration occurs to a large extent of dopaminergic neurons – specifically located in the substantia nigra pars compacta. The pathways discussed above work in conjunction to induce cell death in the neurons. Specifically, the formation of intraneuronal inclusions and chronic inflammation are the largest contributing factors to neuron degeneration. Neurotoxins, especially MPTP, are considered to be the leading cause of neuronal degradation [26].

Hirsch et al. found that a sub-population of dopaminergic neurons, stained by neuromelanin, are more susceptible to degradation. This is due to the fact that MPTP and its metabolite, MPP+ (responsible for neuronal deterioration), bind to neuromelanin [27]. Furthermore, Youn et al. demonstrated that the transduction of PEP-1-heme oxygenase-1 (PEP-1-HO-1) in human neuroblastoma SH-SY5Y cells inhibited the production of reactive oxygen species (*ROS*). Intraperitoneal injection of PEP-1-HO-1 in PD mouse models significantly reduced the toxic effects of MPTP and MPP+. These findings suggest that PEP-1-HO-1 could be a viable agent in the treatment of oxidative stress-induced PD. Both studies exemplify the interdependent nature of PD pathogenesis on specific biological interactions. The onset of PD is incremental as each outlined mechanism appears to be a trigger of sorts for another [28].

Role of Neurotrophic Factors in PD

Due to the fact that PD is a neurodegenerative disorder, one of the most promising directions for therapeutic research is in reviving neurons using growth factors (GFs). GFs are biological compounds that stimulate cellular regeneration and thereby facilitate the process of healing. Although nerve/neural growth factors (*NGF*s) are commonly applied to treat cerebral disorders, a variety of general growth factors have been tested to potentially treat PD [6].

Growth factors function by binding to cellular receptors and subsequently activating cell signaling cascades that regulate mitosis, differentiation and apoptosis. Neurturin (*NRTN*), neurotrophin (*NTR*), epidermal growth factor (EGF), liver growth factor (LGF), insulin-like growth factor 1 (IGF-1), intrastriatal transforming growth factor alpha (TGFα) and fibroblast growth factor 20 (*FGF20*) are a few of the common GFs that have been tested to treat PD. Table 1 summarizes potential growth factors, their intended therapeutic function and existing papers which support the applications of these GFs.

NRTN is a naturally occurring analog of glial cell line-derived neurotrophic factors (*GDNF*s), commonly used to target dopaminergic neurons in the nigrostriatal pathway of PD animal models [29]. Its observed effects include delay of neuron degeneration, selective protection of dopaminergic neurons and general enhancement of neural functioning [30]. Currently, it is also believed that the levels of docosahexaenoic acid (DHA, a major polyunsaturated fatty acid in the brain) alters levels of *NRTN* [31]. Gasmi et al. (2007) conducted animal trials to achieve striatal delivery of *NRTN* by using CERE-120 (an adeno-associated virus type 2 - AAV2). Viral vector-mediated delivery of *NRTN* genes has since been an increasingly targeted mechanism of therapy for PD [32]. Olanow et al. (2015) recently conducted a double-blind, randomized, controlled trial to measure the efficacy of AAV2 as a vehicle for *NRTN* delivery to the substantia nigra and the putamen. Post-mortem testing found that *NRTN* was expressed in the putamen, bilaterally; however, minimal expression was observed in the substantia nigra pars compacta (SNc). Researchers hypothesized that a delayed response to AAV2-*NRTN* was observed due to impaired transport from the putamen to the cell bodies in the SNc, characteristic of PD [5].

NTR was also found to have promising effects in idiopathic cases of PD. Studies have observed that apoptotic cascades in Parkinsonian patients are also correlated with decreased levels of *NTR* [2,33]. Within the CNS, *NTR* functions by improving the efficacy of nigral grafts [34,35]. Haque et al. tested the application of *NTR*4/5 to increase the survival of dopaminergic neurons in ventral mesencephalic tissue grafts. Transplantation of such grafts in the substantia nigra has been a method of treatment for PD. Haque's study found that the infusing of *NTR*4/5 (but not *NTR*3) stimulated fibre growth and enhanced the functionality of the nigral grafts [36]. Furthermore, it is worth noting that the increased efficacy was observed *in vivo*, suggesting that the application of *NTR* to facilitate neuron regeneration and surgical graft integration is a viable option to alleviate PD-associated symptoms in the long term.

Aside from the described *NGF*s, general growth factors, including *EGF* and *LGF*, have also been applied in the context of PD. Iwakura et al. explored the role of *EGF* in PD. EGF exerts neurotrophic activity on dopaminergic neurons in the midbrain. Iwakura's results demonstrated that the expression of *EGF* receptors were downregulated in the post-mortem brains of PD patients. These findings are indicative of *EGF*'s neurotrophic activity being mod-

ulated by afferent signals of dopaminergic neurons [37]. Additionally, *EGF*'s activity is further impaired by neural degeneration that is characteristic of PD, making *EGF* a potential target for therapeutics [38,39].

Gobernado et al. observed neuroprotective activity when LGF was administered, peripherally, in a rat model of PD [40]. LGF is a hepatic mitogen that promotes proliferation of various cell types and facilitates tissue regeneration. Upon peripheral application of LGF to the 6-hydroxydopamine (6-OHDA)-injected region in the left striatum, unilaterally, sprouting of tyrosine hydroxylase-positive terminals and dopamine transporter expression was increased. LGF also stimulated the phosphorylation and regulation of proteins critical for cell survival - including Bcl2 and Akt. Due to the partial protection LGF provides dopaminergic neurons from 6-OHDA neurotoxicity and alleviation of motor-based symptoms in the PD rat models, along with improved efficacy of nigral grafts, LGF could be administered to treat PD [7].

Insulin-like growth factor 1 (IGF-1) provides neuroprotective effects through its anti-apoptotic properties that mainly target the endoplasmic reticulum in neurons [41,42]. Godau et al. found that IGF-1 could be a serum marker for early PD and could therefore play a critical role in earlier diagnosis of PD [43]. Additionally, it was also observed that IGF-1 may assist in neuronal protection from toxic substances that are characteristic of PD, particularly DA-induced toxicity [44].

Intrastriatal delivery of transforming growth factor alpha (TGF-α) has been shown to significantly stimulate the proliferation and substantial migratory waves in dopamine-denervated rats [45]. Furthermore, intrabrain transplantation of TGF-β1 gradually improved the overall condition of parkinsonian rats through striatal reinnveration and increase of dopamine levels in the grafted striatum [46].

Fibroblast growth factor 20 (*FGF20*) is substantially expressed in the substantia nigra and is believed to play a crucial role in the protection of dopaminergic neurons [47]. Specifically, it was found that the *FGF20* gene rs1721100 polymorphism is associated with an elevated PD risk [48,49]. Mizuta et. al also found that *FGF20* had a significant presence in the *SNCA* homozygote (risk allele). *SNCA* is the gene that codes for alpha synuclein (the primary constituent of Lewy Bodies) [50]. *FGF20* and *SNCA* work synergistically, suggesting that *FGF20* could alleviate PD symptoms by interfering with the mechanism of Lewy Body formation.

AN INTRODUCTION TO RETROVIRAL-BASED GENE DELIVERY SYSTEMS

Retroviral vector-mediated gene delivery is an area of gene therapy that has gained increasing attention over the past decade to deliver genes to target cells using viral particles as vehicles [51]. Lentiviruses belong to the Retroviridae family of viruses and are especially useful in introducing genes into the host DNA. An example of a lentivirus genome that has been widely exploited is the human immunodeficiency virus (HIV) genome that consists of structural genes called *gag*, *pol* and *env* that package the viral core, regulatory genes named *tat* and *rev* that are involved in viral replication as well as accessory genes known as *vif*, *vpr*, *vpu*, and *nef* involved in viral growth and propagation *in vivo* [52]. Once the target genes are packaged into these viral vectors, they convert their single stranded RNA into a double stranded DNA that can stably integrate into the host genome. The integrated vector, called the provirus, undergoes replication and transcription in the host genome producing the viral mRNAs and the packaged RNA as well [53]. The advantage of using lentiviruses is that they allow for the stable integration of genetic material in non-dividing, terminally differentiated cells [53,54]. Specifically, lentiviral-based vector delivery systems have been used *in vivo* to target various diseases, ranging from blood-borne diseases like X-linked severe combined immunodeficiency (SCID-X1) [53], skeletal muscle disorders like Duchenne muscular dystrophy [55], cancer immunotherapy, [51] to neurodegenerative diseases such as Parkinson's disease [8], among others.

Development of Safer, Viable Vector Systems

However, since the initial development of lentiviral-based vector delivery systems, both vector performance and safety issues have risen time and time again. Because there is an active and dynamic contact with the host genome, threats of oncogene activation and insertional mutagenesis through the activation of non-specific endogenous promoters encroach vector applicability [56,53,57]. To address these concerns, research in viral vector development has picked up and delivered safer options, specifically exploiting the structure of the viral particle. The human immunodeficiency virus (HIV) has been extensively studied and the genome has been manipulated in order to safely use this virus as a research tool. Genes encoding the various viral components were either expressed through separate plasmid constructs, removed, modified in orientation/conformation, repressed (i.e., self-inactivating constructs) or adapted from other viruses to tone down virulence while still retaining adequate efficacy [56,57]. Adapting a vector system from a primate model confers the added advantage of potentially reducing an immune response.

The first generation development of these viruses involved a different viral envelope than HIV, specifically the G protein of the vesicular stomatitis virus (VSV-G) adapted to coat the virus. Known as pseudotyping, this can be exploited as a targeting mechanism if the coat is genetically modified to bind specific proteins/receptors of a tissue subpopulation. The second generation vectors limited the vector packaging component to four essential genes, namely *gag*, *pol*, *tat* and *rev*. The third generation

Table 2. An overview of clinical trials testing viral vector delivery

Viral Vector and Gene	Limitations	Proposed Improvements	References
AAV-hDAAC (human L-amino acid decarboxylase)	Lack of control for result comparison. Non-blinded analysis increased difficulty of result interpretation. DA levels not measured.	Progress is limited as the results did not show significant improvement. A well-defined control and double-blind analysis would improve result interpretation.	Eberling et al., (2008)
CERE-120 (AAV serotype 2 - *NRTN*)	Secondary measures of motor function did not show significant improvement.	Good tolerance of the treatment, without any clinically significant adverse effects was observed. More specific facets of measuring motor function could be employed.	Marks et al., (2008)
AAV2-*NRTN*	Many patients developed severe adverse reactions due to surgical procedure. No signficiant change in comparison with control.	Demonstrated that gene therapy results in long-term gene expression. Increased sterilization and minimally invasive surgical procedures would reduce design limitations.	Marks et al., (2010)
AAV2-*GAD*	Mild adverse reactions occurred for most patients. One severe adverse reaction was reported.	Majority of PD patients receiving the AAV2-*GAD* treatment had a significant improvement from their baseline UPDRS score. Adverse reactions can be overcome by introducing more rigorous evaluation of treatment.	LeWitt et al., (2011)
ProSavin	Cases of mild on-medication dyskinesia and on-off phenomena reported.	Reduction in resting tremors and increased motor control found. Treatment was well-tolerated and safe. Further research should be conducted to determine potential sources of on-off phenomena.	Palif et al., (2014)

of vectors, placing *rev* in a *trans* conformation, allows the production of high titre *gag* and *pol* thus dispensing *tat* as well [52]. Thus, vectors, initially derived from the human immunodeficiency virus (HIV), have been modified with the removal of regulatory and accessory genes that encode virulence factors [51]. Hence, a replication-defective lentiviral particle has been created with a viral core consisting of structural proteins and enzymes, an envelope of an unrelated virus and the lentiviral genome. Over the years, lentiviral systems have been developed to offer certain advantages over other competing viral vector systems such as the ability to transduce dividing as well as non-dividing cells, demonstrate long-term, stable gene expression, and safely infect target cells at a high efficiency [58].

Exploiting this efficient delivery system for cell-based therapeutics is an emerging field. Using the lentiviral-based gene therapy as a tool, one can exploit the potential of engineering cells to sense, "process," and respond to a dynamic environment [59]. It is well established that in HIV infected individuals, the virus does reach the CNS via infected macrophages that cross the blood brain barrier [53]. Although the mechanism of CNS pathology continues to be explored, it is known that the *env* and *tat* proteins cause neurotoxicity *in vitro* [53]. Therefore, viral vector technology aids one to develop a recombinant, replication-deficient viral particle that delivers a therapeutic gene to a specific cell population efficiently.

VECTOR TARGETED DELIVERY TO THE CNS

Whereas an *ex vivo* approach is aimed at modifying the target cell population outside of the body and then reintroducing the cells via implantation, an *in vivo* approach uses vector delivery systems such as the lentiviral vector system to deliver the therapeutic gene allowing for the direct manipulation and establishment of stable, long-term control in a non-dividing neuronal population by permanently integrating into the host cell population. Lentiviral vectors are particularly advantageous due to their large cloning capacity of 8 to 10 kilobase pairs. The therapeutic gene can act on various levels such as binding

and inhibiting the mRNA of the dysfunctional, target gene or binding the protein itself [60]. Going one step further, it is possible to achieve temporal and spatial control of these vectors using transgenic or knock-out/knock-in models. Ideally, the proposed viral vector should be specifically targeted to the host population and not generate an immunological response.

Practical elements to consider when generating a targeted viral vector to the brain include selection of viral serotype (i.e., groups sharing specific surface molecules) and injection dose and site. In particular, pseudotyping with various viruses such as the VSV and Mokola virus leads to CNS transduction [61]. The use of a chimeric viral vector system was recently applied to cure two distinct types of brain tumors in mice [62]. The virus VSV-G, with its broad tropism, readily infects tumor cells but causes widespread neurotoxicity in the brain, even when expression is attenuated via mutations. The viral vector VSV-LASV-GPC, encoding the wildtype VSV from the Indiana serotype for the G protein, was fused with the Lassa fever virus glycoprotein gene with a GFP reporter gene engineered on the C-terminus for visualization. When tested, this chimeric vector showed reduced infection of nomal glia and neuronal cells versus tumor cells (i.e., gliomas) creating *in vivo* target specificity. Intracranial or intravenuous (tail-vein) injections in mouse models showed target specificity as VSV targeted brain tumors. Normal cells were protected due to the activation of type I interferon (a large group of interferon proteins that regulate innate immune system activity) as compared to tumor cells [62]. Safe vector dosages range from 10^2 to 10^6 transducing viral units [62,63]. Taking such practical considerations into effect can increase the efficicacy of the target vector delivery system.

An Overview of NGF*-Based Clinical Trials for Parkinson's Disease*

Currently, the vast majority of clinical trials for Parkinson's disease employ the use of adeno-associated viruses to deliver neurotropic factors in order to provide neurotrophic support and have not progressed beyond Phase II [64–68]. Although the lentiviral approach has shown relative success when applied to primate model systems [69–71] in the past, no adequate results were reported from ongoing clinical trials. Along with certain methodological limitations, as outlined in Table 2, perhaps the drawbacks observed in clinical trials is due to our limited understanding of lentiviral effects *in vivo*. Furthermore, the measurement of long term effects of stable neurotrophic factors has not been incorporated, in a rigorous manner, in a majority of the clinical trials outlined below.

Eberling et. al conducted bilateral infusion of an AAV containing the human aromatic L-amino acid decarboxylase (hDAAC) gene, into the putamen of patients with moderate to advanced levels of PD [64]. Low doses of the AAV-hDAAC injection produced an average of 30 percent increase in fluoro-L-M-tyrosine (FMT) (an *in vivo* measurement of gene expression). A primary downfall of the study was the fact that a control was not utilized. Furthermore, the study itself states that "nonblinded analyses make interpretation difficult." The transfection of hDAAC into nondegenerating striatal neurons is expected to convert low doses of L-dopa (a precursor of dopamine) into high levels of DA. The study, however, was unable to directly quantify levels of DA, creating questionable results that must be interpreted cautiously [64].

Marks et. al (2008) initially conducted a phase I clinical trial to determine the safety and tolerability of CERE-120 (AAV serotype 2 – *NRTN*). Patients with idiopathic PD received bilateral, intraputaminal injections of the vector. The results primarily showed good tolerance of the treatment, without any clinically significant adverse effects within a year after injection. The study claims that several "secondary measures of motor function" showed improvement – including a mean improvement in the off-medication motor subscore; however, the improvements were not significant [66].

Marks et. al (2010) also conducted a double-blind, randomized, controlled trial for the gene delivery of AAV2-*NRTN*. A cohort of advanced PD patients were randomly assigned to receive either AAV2-*NRTN* (injected bilaterally into the putamen) or a sham surgery. The results found that there was no significant difference between patients treated with AAV2-*NRTN* and the control group. Furthermore, 13 out of 38 patients treated with AAV2-*NRTN* developed severe adverse reactions (mainly due to the surgical process). Although the results themselves did not provide any significant benefit, the study was able to show that gene transfer enables long-term gene expression. However, this property means that patients must be followed-up frequently upon receipt of the procedure [65].

The primary drawback of the above studies mainly relates to the surgical techniques and injection mechanisms employed to deliver the lentiviral vector. A significant proportion of adverse reactions (e.g. intracranial hemorrhaging) were believed to have occurred as a result of surgical procedures [72]. Currently, procedures include vertical administration of the vector from the dorsal surface of the brain and identification of intraputaminal targets using the Leksell stereotactic frame and MRI guidance [73]. These procedures, however, can cause unintended effects without employing a meticulous and methodical approach. For example, increased precision can be achieved using localization software, enabling more efficient targeting.

Nevertheless, despite the lack of success with pre-existing lentiviral-mediated delivery of neurotrophic factors, ProSavin clinical trials show a promising avenue for future development. ProSavin is an experimental drug that uses a lentivector delivery system to transfer genes to the

striatum. Palfi et al. (2014) bilaterally injected ProSavin into the putamen of PD patients. A series of three different doses were utilized. In the first 12 months, mild drug-related adverse reactions were reported, mainly consisting of on-medication dyskinesia and on-off phenomena. Results suggested that ProSavin administration was safe and well-tolerated. Furthermore, motor improvement, including reduced resting tremors and increased motor control, were observed in all patients [73].

LeWitt et. al (2011) attempted to compare the efficacy of gene transfer of glutamic acid decarboxylase (*GAD*) with sham surgery. Patients with progressive levodopa-responsive PD received bilateral injection of AAV2-*GAD* to the subthalmic nuceleus. It was hypothesized that similar to animal models, *GAD* would improve basal ganglia function. The study also showed promising results as a majority of PD patients receiving the AAV2-*GAD* treatment had a significant improvement from their baseline unified PD rating scale (UPDRS) score. The study also showed safety of the treatment, as the most common adverse reactions were mild, including nausea and headaches [68].

We propose that *in vivo* gene therapy, primarily using lentiviral vehicles, is a promising therapeutic approach, despite the inadequate results produced by existing clinical trials. Primary advantages of this approach include permanent changes of the neuron's genome. Most of the wild-type genome of the virus is deleted, resulting in minimal toxicity. Additionally, invasiveness of the therapy is decreased as only one injection to the site is required, contrary to multiple injections that would result if the neuron was unable to produce its own neurotrophic factors. Currently, most clinical trials employ bilateral injection of the vector to the putamen or the striatum. Perhaps novel injection techniques can be determined to increase the efficacy of lentiviral vectors and to reduce surgery-associated adverse events [72,74]. Table 2 shows a summary of clinical trials and a brief analysis of their successes and drawbacks.

An Example of a Lentiviral-Based Delivery System

Delivery mechanisms for gene therapy differ in their targeting scope with some targeting widespread target populations such as direct injection, while others are more specific to certain subpopulations such as pseudotyping (for example, brain region vs. glial cell population). The convention is to use direct injection protocols either into the retina or brain that bypass the blood brain barrier [74]. Other than being an invasive technique, a lower transfection efficiency and the need for high viral titres offset the potential applicability of direct injection. On the other hand, pseudotyping can achieve acute target specificity with viral coats that can easily transduce specific cell types such as haematopoetic stem cells (using Feline leukemia virus) and neuronal cells (Ross river virus) [75]. The most widely used glycoprotein for pseudotyping is VSV-G due to its broad tropism and stability [76]. This broad tropism is achieved by the glycoprotein attaching to a ubiquitous cell receptor. However, to achieve cellular specificity, glycoproteins exist that are targeted to specific cell types or organs - for example, targeting Ross River virus (RRV) to Kupffer cells of the liver, Ebola virus to lung, the lymphocytic choriomeningitis virus (LCMV) to pancreatic islet cells, the Mokola virus to cardiomyocytes of muscle tissue and the Feline endogenous retrovirus (RD114) to the hematopoteic system among others [76]. Going one step further, instead of adopting these coats from existing viruses, it is possible to engineer these coats to obtain cell-type specificity. Such an approach allows one to modify the viral surface with proteins (i.e., cell-specific peptides or antibodies). In addition, using mammalian promoters such as synapsin 1 and glial fibrillary acidic protein (*GFAP*) to drive expression of lentiviral vectors to targets have been used previously [77].

In the lentiviral transgene cassete, we propose to include a microglia-specific promoter and an anti-α-synuclein antibody. Candidate microglial specific promoters that are well characterized in humans include *NGF* from - 600 to + 250 nucleotides [75] and *BDNF* exon III from + 2623 to + 3028 nucleotides [77,78]. A candidate anti-α-synuclein antibody is *PER1* under the control of the strong human cytomegalovirus (CMV) promoter to initiate high level stable mammalian expression. Specifically, *PER1* is a synthetic antibody synthesized against residues 11-34 of α-synuclein and thus exclusively recognizes the "α" isoform of the synuclein protein. While the microglia specific promoter provides cellular specificity, the antibody will provide therapeutic benefits. To assess any benefits, the amount of debris (i.e., unfolded protein) removed from the CNS should be determined periodically every few months. Using the doxycycline regulatory system [18,78] and the fluorescent cassette strategy [79], the promoter/gene functionality needs to be controlled and validated *in vitro* before moving on to mouse models.

Previously, Recchia et al. (2007) were able to induce some of the prevalent symptoms of PD in rat models through the intranigral injection of TAT-α-synA30P (a transduced protein construct). The usage of a transduction domain derived from HIV enabled the construct to diffuse through the neuronal membrane, resulting in selective dopaminergic loss and long-term motor debilities. Particularly, it was found that the novel method of α-synulcein integration induced symptoms associated with the early stages of PD in rat models [80]. Using these findings and two other Parkinson's disease models with nigral synucleinopathy, one can measure the efficacy of the delivery system *in vivo*, when employed intravenously. Whereas the AAV-α-synuclein viral model mimics the disease genotype, the inducible drug-based MPTP model illustrates the disease phenotype. Specifically, the AAV-α-synuclein viral model activates the adaptive immune response stimulating microglial proliferation [81]. On the

other hand, the MPTP model causes nigrostriatal neuronal loss leading to Parkinson's disease motor symptoms such as rigidity, tremor, and gait and posture abnormality.

CONCLUDING REMARKS

Herein, we have provided a potentially applicable model system wherein a lentiviral-based delivery tool can be engineered to target and alleviate Parkinson disease symptoms. Using this approach ensures that viral vectors can transduce changes in non-dividing neuronal cells of the brain. The delivery of neurotrophic factors to the brain alleviates the inflammatory stress induced by the CNS innate immune system. With long-term stability and integration, the therapeutic potential of vectors are significant. Nevertheless, the long term effects of expressing neurotrophic factors needs to be readily assessed alongside to avoid other detrimental side effects, such as overexpression of genes. Potential methods to assess the future effects of stably expressed neurotrophins include monitoring immunoreactivity and utilizing staining techniques to observe and predict the occurrences of on-target and off-target effects [82,83]. Although issues of vector genotoxicity may still exist and the field may be far from addressing patient-specific needs, with the development of cell-specific gene therapy techniques, we are establishing a framework on which to build a comprehensive therapeutic approach.

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