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Scientific rationale for the development of gene therapy strategies for Parkinson's disease

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Abbreviations: 6-OHDA: 6-hydroxydopamine, AADC: aromatic acid decarboxylase, COMT: catechol-*O*-methyltransferase, DBS: deep brain stimulation, DA: dopamine, DAT: dopamine transporter, EIAV: equine infectious anemia virus, FDG: fluorodeoxyglucose, FMT: fluoro-L-m-tyrosine, GABA: gamma-aminobutyric acid, GDNF: glial derived neurotrophic factor, GFR α : glycosyl-phosphatidylinositol (GPI)-linked subunit, GP: globus pallidus, GPi: internal segment of globus pallidus, Glu: glutamate, GAD: glutamic acid decarboxylase, GFP: Green Fluorescent Protein, GCH1: GTP cyclohydrolase 1, ICV: intracerebroventricular, IgSP: immunoglobulin heavy-chain signal peptide, L-DOPA: 3,4-dihydroxyphenylalanine, MPTP: 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine, NGF: nerve growth factor, NTN: neurturin, PD: Parkinson's disease, PDCP: Parkinson's disease-related cognitive pattern, PDRP: Parkinson's disease-related covariance pattern, PPRS: Primate Parkinsonian Rating Scale, PET: Positron Emission Tomography, rAAV: recombinant adeno-associated virus, rAd: recombinant adenovirus, rLV: recombinant lentivirus, BH4: tetrahydrobiopterin 5HT: serotonin, STN: subthalamic nucleus, SNr: substantia nigra pars reticulata, TH: tyrosine hydroxylase, UPDRS: Unified Parkinson's Disease Rating Scale, VG: vector genomes

Abstract

The ever-evolving understanding of the neuronal systems involved in Parkinson's disease together with the recent advances in recombinant viral vector technology has lead to the development of several gene therapy applications that are now entering into clinical testing phase. To date, four fundamentally different approaches have been pursued utilizing recombinant adeno-associated virus and lentiviruses as vectors for delivery. These strategies aim either to restore the lost brain functions by substitution of enzymes critical for synthesis of neurotransmitters or neurotrophic factors as a means to boost the function of remaining neurons in the diseased brain. In this review we discuss the differences in mechanism of action and describe the scientific rationale behind the currently tested gene therapy approaches for Parkinson's disease in some detail and pinpoint their individual unique strengths and weaknesses.

Introduction

The first reported clinical application of gene therapy was conducted almost 20 years ago using *ex vivo* gene transfer by retroviral transduction as a means to deliver functional lymphocytes with corrected adenosine deaminase activity to a young girl with severe combined immunodeficiency [1]. Although the safety of this particular approach (and even gene therapy as a whole) was later questioned, development of novel, non-integrating, recombinant viral vectors and advances in the fields of immunology and virology have led to clinical protocols that are now considered sufficiently safe for *in vivo* gene delivery in several diseases that affect different tissue types and organs. One field where the gene therapy approach has caught attention is the treatment of neurodegenerative disorders. As we will detail in this review, Parkinson's disease (PD), in particular, is a well-suited target for gene therapy for a number of reasons and not surprisingly, there are currently four ongoing or completed clinical trials aimed for therapeutic relief in PD utilizing viral vector-mediated gene delivery in the brain.

The general attitude toward clinical gene therapy during the last decade would probably be best described as careful optimism. Many have seen it as an interesting technique for proof-of-concept studies, but few have viewed it as a viable strategy for routine use in a broader clinical setting. Previously, any therapy that required surgical intervention was considered impractical from the points of safety, feasibility and/or economical and human resources. The success of deep brain stimulation (DBS) and its broad application in PD therapy has, however, shown that such requirements are not insurmountable.

Viral vector-mediated gene transfer provides some unique advantages that can make it preferable over traditional pharmacotherapy in neurological disorders. A number of traits of PD illustrates this distinction well: Firstly, gene therapy enables delivery of complex molecules or enzymes to specific regions of the brain at constant rates. This provides an opportunity to explore novel mechanisms for therapy and modulation of individual pathways. The bio-distribution of the virally expressed therapeutic protein can be optimized specifically for each patient by controlling the placement of injection, volume and concentration of the vector delivered. This can circumvent a number of adverse effects otherwise linked to the same therapeutic approach, when applied systemically. In many neurological disorders, neuronal populations located in different nuclei in the brain are effected to varying extent. A systemically delivered drug might therefore normalize the system in some regions of the brain but at the same time over-stimulate other neurons of the same kind in regions less affected by the disease process.

Secondly, the socioeconomic aspects of gene therapy based medications could be generally more favorable than might be assumed. The increase in life expectancy and reduced birth rate seen in most countries of the industrialized world during the last decades has resulted in a rapid increase in the aged fraction of the population. Therefore, governments face dramatic increases in healthcare costs due to age-related diseases. One of the categories that put significant strain on the budget is debilitating, chronic diseases that effect people at working age such as PD, which exhibits an average onset age of 58 years in the US. With only a slight reduction in life expectancy due to the disease [2], most patients would require therapy for at least 20 years and as 5-10% of them develop symptoms before the age of 50, it can in many cases be significantly more. While the introduction of novel compounds such as long

lasting dopamine (DA) agonists and various enzyme inhibitors into the routine arsenal of medication might have improved the duration of good response to pharmacotherapy, the costs of treatment per patient and year have increased substantially. Gene therapy treatments, on the other hand, are based on a single intervention principle, resulting in stable and long-term (maybe life-long) expression of the therapeutic molecule in the brain. Reduction in the requirement for medication in turn might reduce the financial burden of the treatment to society.

Thus far, three clinical trials targeting PD patients have been completed and published as peer reviewed scientific papers [3-6]. All three studies were conducted using recombinant adeno-associated virus serotype 2 (rAAV2) vectors with the transgene expressed under a ubiquitous promoter. Furthermore, all three studies aimed to test the safety, tolerability, and preliminary efficacy of the proposed strategy in an open-label, phase I clinical trial design. The tested therapeutic molecules and the principle mechanism of action, however, vary greatly between them.

In this review, we aim to describe in detail the current clinical approaches for gene therapy in PD (where information is available), the rationale and pre-clinical evidence for the chosen paths. We have chosen not to cover the choice of viral vectors for the experimental and clinical applications here as this topic has been reviewed detailed elsewhere [7, 8].

Principles and the rationale for the strategies tested in the first gene therapy trials in Parkinson's disease

The molecular pathways and target structures in the recently published clinical trials were chosen based a number of pre-clinical and clinical observations, some of which

are well supported, whereas others, in our opinion, are not sufficiently documented. Based on their functional target and mechanism of action these trials can be reviewed under three categories: (1) restoration of DA synthesis in the dorsal striatum; (2) modulation of activity in the basal ganglia downstream of the striatum; and (3) modification of disease progression by neuroprotection.

Restoration of DA synthesis capacity

Since the discovery of DA as a neurotransmitter and its involvement in Parkinson's disease [9-12], the focus of pharmacological therapies has been on restoring the DAergic tone in the brain. The reconstitution of striatal DA via peripheral L-DOPA (3,4-dihydroxyphenylalanine), combined with peripheral decarboxylase inhibitors, has proven itself as one of the most successful therapies for a neurological disorder [13] and became the gold-standard for PD pharmacotherapy [14, 15]. In the initial years, when motor complications are the predominant symptoms of the disease, L-DOPA medication provides excellent symptomatic relief and can greatly improve the quality of life for the patient. However, long-term treatment with L-DOPA is not without limitations and adverse events, which inevitably emerge in more than 80% of all PD patients within the first 10 years from disease onset [16]. With disease progression non-dopaminergic neural systems become affected, which cannot be effectively alleviated with L-DOPA. In addition, some patients may show signs of involuntary movements, so-called dyskinesias, already in the first few years. Furthermore, L-DOPA can lead to other adverse events can include for instance hypotension, sexual dysfunction or psychiatric side effects [17, 18].

Pharmacokinetics and biodistribution studies suggest large fluctuations in the serum levels of L-DOPA after oral administration [19, 20]. Thus, it was hypothesized

that the development of dyskinesias might be a result of the fluctuations in DA concentrations at the synaptic sites in the denervated striatum. This hypothesis is supported by clinical data showing that continuous infusion of L-DOPA, delivered either intravenously or via duodenal pump can significantly reduce the occurrence and magnitude of dyskinesias and decrease the daily “off” time [21-23]. Similar results have been reported with intravenous and duodenal infusion of apomorphine, a non-selective DA receptor agonist [24]. Continuous DA stimulation using L-DOPA infusion pumps or DA receptor agonists is a valuable addition to the plethora of alternatives used to control “on–off” fluctuations and dyskinesias in late stage patients. However, these approaches do not address and in some cases worsen several of the complications. First, systemically delivered DAergic drugs reach the whole brain at high concentration. This is clearly not the best approach since not all brain regions suffer from DAergic degeneration to the same extent. For example, the requirement of additional DAergic tone might be substantially less in the limbic and cortical areas than the severely affected striatum. Thus, in this mode of treatment these regions might be constantly over-stimulated with high DA tone, which is adjusted primarily for relief of motor symptoms in the patients. Therefore, other treatment approaches that can locally enhance the DA concentrations in the striatum could prove to be more beneficial and limit the occurrence and severity of side effects to levels not achievable with the currently available treatment modalities.

At least three major gene therapy strategies have been developed to synthesize DA locally in the brain (graphically represented in Fig. 1). The main factor that differs between these approaches is the interpretation of which enzymes are necessary and sufficient to express ectopically in the target area of the brain to reconstitute the DA synthesis capacity. It is widely accepted that the Tyrosine hydroxylase (TH)

enzyme activity is significantly reduced in the parkinsonian striatum, severely compromising the rate of synthesis of DOPA from tyrosine. Thus, it is clear that striatal DOPA must be replaced. Whether the amount of aromatic acid decarboxylase (AADC) enzyme available in the diseased brain is sufficient for synthesis of DA in the appropriate target regions, however, is a matter of debate. The AADC enzyme is present in the striatum, not only in DAergic axons but also in serotonergic terminals [25, 26], but it has been shown that the levels are decreased in the striatum of PD patients. The reported level of residual AADC activity is variable between patients and also between studies. It could be as low as 5% in the most severely affected cases, and usually larger decreases are found in the putamen than the caudate nucleus [27, 28].

Pro-drug approach for enhanced DA synthesis

If the levels of AADC enzyme were increased or even restored to normal levels selectively in the striatum, then a larger fraction of the total systemic L-DOPA would be converted to DA in this part of the brain. As a result, the dose of oral L-DOPA could be decreased with maintained efficacy, whereas the effects due to extrastriatal DA synthesis may be minimized. This strategy is also known as the pro-drug approach. The first proof-of-principle for this therapy was demonstrated in primates with a unilateral MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) lesion that received an injection of rAAV2 vectors coding for the human AADC gene. These animals showed increased conversion efficacy of peripheral L-DOPA to DA as seen by biochemical analysis of tissue punches from the transduced striatum [29].

When applied to MPTP lesioned non-human primates, rAAV mediated AADC delivery resulted in an increased accumulation of [^{18}F]-MT (fluoro-L-m-tyrosine), in

the transduced striatum compared to lesion controls [29]. FMT is an L-DOPA analog that cannot be methylated by catechol-*O*-methyltransferase (COMT) in peripheral organs and erythrocytes but is still a valid substrate for the AADC enzyme. Thus, the background signal in the brain is less than that of [¹⁸F]-DOPA but with maintained specificity for neurons that display decarboxylase activity [30]. [¹⁸F]-MT retention in the brain correlates well with the total concentration of AADC (present in dopamine, serotonin and norepinephrine containing neurons) but is not dependent on vesicular monoamine transporter (VMAT2) function [30]. In a follow-up study, Bankiewicz and colleagues presented long-term behavioral improvement and *in vivo* AADC enzyme activity for up to 6 years after rAAV-AADC transduction of MPTP treated monkeys [31]. The animals displayed a 50 % improvement in the clinical rating scale after a single injection of L-DOPA at a dose that was not sufficient to induce a significant improvement in control vector injected animals. However the duration of action for peripheral L-DOPA in these rAAV-AADC transduced primates was not reported. Nevertheless, the behavioral effects were coupled with a normalized striatal [¹⁸F]-MT uptake that was stable for the full duration of the study [31].

The pro-drug approach is often touted as attractive from a safety point of view because it enables peripheral regulation. In case of adverse events, the peripheral supply of L-DOPA can be discontinued and the ectopic AADC enzyme would be rendered inactive. However, one point of concern is if the ectopic release of DA from transduced cells were too efficient, the extracellular levels of DA after an oral L-DOPA intake might increase too rapidly. Thus the therapy could increase the fluctuations of DA supply and aggravate dyskinesias. In fact, it has been shown in primates that even AADC over-expression alone can potentiate L-DOPA-induced dyskinesias if the transduction is heterogeneous [32]. If this were to happen, the daily

L-DOPA dose would have to be decreased or even discontinued until the adverse events disappear. However, as oral L-DOPA is the main, most efficacious pharmacotherapy for the patients, this would leave them worse off than before the intervention.

A phase I, clinical safety trial utilizing rAAV mediated gene delivery of AADC as a therapy for PD is currently ongoing. In this study, ten patients with advanced PD (Hoehn and Yahr Stage III to IV) were injected bilaterally into the postcommisural putamen with an rAAV2-AADC vector (ClinicalTrials.gov registry number NCT00229736). The first group of five patients has been operated to date. These patients received 9×10^{10} vector genomes (vg) of the rAAV2-AADC vector and were followed for 6 months post injection with evaluation using both the Unified Parkinson's Disease Rating Scale (UPDRS) and Positron Emission Tomography (PET) imaging utilizing [^{18}F]-MT. The treatment was well tolerated and induced a robust increase in [^{18}F]-MT uptake 6 months post injection. The patients, however, did not display any improvement in UPDRS "on" score i.e. on oral L-DOPA medication but improved off medication [4]. These results were somewhat contrary to the assumed function as the over-expressed AADC was expected to mediate its functional effects via enhanced conversion of peripheral L-DOPA to DA.

Dopamine replacement

The alternative to the pro-drug approach is to reconstitute all the enzymes that are required for DA synthesis in the parkinsonian striatum. In the normal brain, the DOPA substrate used by the AADC enzyme to synthesize DA is generated from dietary tyrosine by the TH enzyme. This enzymatic conversion is very inefficient in the absence of the co-factor tetrahydrobiopterin (BH₄) [33-35]. As a large fraction of

the striatal BH4 content is lost with DA denervation, the availability of this co-factor limits the efficacy of the DOPA synthesis from ectopically expressed TH [36, 37]. The rate-limiting enzyme in the production of BH4 is the conversion of GTP to dihydroneopterin triphosphate by the GTP cyclohydrolase 1 (GCH1) enzyme. Further conversion of this intermediate to BH4 is then catalyzed by two ubiquitously expressed enzymes, 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. Therefore, co-transduction of TH and GCH1 genes is sufficient to sustain high levels of DOPA synthesis in various cell-types both *in vitro* and *in vivo* (see [8] for a detailed review of this topic). These two enzymes can then be combined with AADC to provide a 3-enzyme replacement strategy for direct synthesis of DA in the transduced cell in the brain. Shen and colleagues demonstrated that *in vivo* gene transfer using a mixture of three rAAV vectors coding for TH, GCH1 or AADC can induce DA synthesis in the DA denervated rodent striatum and that this ectopic DA can reduce apomorphine induced rotation by up to 80% for at least 12 months [38]. The same mixture of rAAV vectors were later applied to parkinsonian cynomolgus monkeys where the animals were reported to improve by up to 64% in the Primate Parkinsonian Rating Scale (PPRS) at 2 weeks post transduction and remained stable throughout the study up to 10 months [39, 40].

Ectopic DA production in striatal cells, however, raises concerns due to the fact that the DA synthesis is localized to cells that have no vesicular storage and release mechanism for this neurotransmitter (Fig. 1b). The first problem that needs to be resolved in this scenario is the strong negative feedback of free cytosolic DA on the TH enzyme both by forming an inhibitory complex with ferric iron (Fe³⁺) and by competitive inhibition of the BH4 substrate binding within the active site of the enzyme (reviewed in detail by Kumar and Vrana [41]). This limitation was

demonstrated by Kang and collaborators in an experiment comparing primary fibroblasts, transduced either with GCH1 and TH, AADC alone or a combination of all three genes [42]. DA synthesis, both in vitro and in the denervated rodent striatum, was significantly reduced when the primary fibroblasts expressed decarboxylase activity in the same cell as the TH activity compared to a setting where these two functions were separated into different cell populations. The inhibition of the TH enzyme can be alleviated by phosphorylation of Serine 40 by reducing the K_m for the BH₄ cofactor and increasing the inhibitory constant (K_i) of DA [43]. It is also known that the enzymatic properties remain and are even slightly enhanced after digestion of the first 158 amino acids of the TH enzyme [44, 45] Thus, the truncated form of the TH enzyme (tTH) that lack the regulatory N-terminal fragment becomes constitutively active regardless of cytosolic DA possibly due to decreased negative feedback [46].

The equine infectious anemia virus (EIAV) was used as a vector platform to carry a tricistronic construct encoding for tTH in combination with the GCH1 and AADC genes[47]. Injection of this multicistronic vector into the striatum of hemiparkinsonian rats resulted in a partial decrease in apomorphine induced rotation but did not result in any detectable increase in striatal DA levels. These results were considered as sufficient basis for a continued development of this vector as a product for clinical testing (ProSavin, Oxford Biomedica, UK). In follow-up studies performed by Oxford Biomedica, the ProSavin vector was injected into MPTP lesioned primates. The company reported in a press release that, at repeated time-points up to 15 months after the vector injection, the monkeys improved their motor performance significantly [48]. With these results, a phase I/II clinical trial has now been initiated in patients with PD [49].

Elevated cytosolic DA levels has been hypothesized to play a critical role in nigral degeneration in PD [50]. Therefore, uncontrolled DA synthesis in cells that lack storage and release mechanisms raises concerns for the long-term implication of ectopic DA accumulation. In fact a recent study in a transgenic mouse model where the DA transporter, DAT, was ectopically expressed in the forebrain cells illustrated this very nicely [51]. The net effect in this model system is that DA released from the pre-synaptic DAergic neuron can be internalized, not only by pre-synaptic terminals (normal re-uptake mechanism) but also by post-synaptic cells in the striatum. As the striatal neuron lacks storage and release mechanisms, DA accumulates in the cytoplasm of these cells resulting in severe oxidative stress, leading to neuronal loss and premature lethality.

Continuous DOPA delivery strategy

An alternative approach to viral mediated DA delivery is the continuous DOPA delivery strategy. Although those two concepts might, on the surface, seem like variations on the same theme, they differ significantly on a number of important points. Continuous DOPA delivery using viral vector-mediated gene transfer relies on endogenous AADC activity for synthesis of DA locally in the brain. Two major sources of AADC in the striatum are the DA and serotonin (5HT) terminals. Thus in the parkinsonian brain the remaining DA axons and the serotonergic terminals are the two most likely places where for conversion to (and release of) DA takes place. As the disease progress, it is anticipated that fewer and fewer DA terminals will remain. Nevertheless, the serotonergic denervation of the striatum is significantly less than the DAergic one in PD patients and thus it may remain as a reliable long-term source in majority of the patients [52]. By separating the L-DOPA synthesis (taking place in transduced striatal neurons) and DA synthesis and release (taking place in DA and

5HT terminals), a number of the afore-mentioned potential complications can be avoided (Fig. 1c) i.e., the need for expression of a constitutively active truncated TH enzyme and the risks associated with intracytoplasmic accumulation of DA in striatal GABA (Gamma-aminobutyric acid) neurons.

The initial studies in this area were instrumental in establishing that not only TH but also BH4 (or the GCH1 gene) had to be provided for optimal DOPA synthesis in the parkinsonian brain. In the first study Corti *et al.*, used recombinant Adenoviral (rAd) vectors to deliver the TH gene. When the animals were injected systemically with high levels of BH4, a 10-15 fold increase was detected in DOPA levels in the striatal tissue of the rAd-TH treated rats [53]. In a second study, DOPA synthesis after co-transduction of rAAV2-TH and rAAV2-GCH1 was shown to be comparable to that of animals receiving the single rAAV2-TH vector combined with reverse microdialysis that delivered BH4 directly to striatal neurons [54]. The combined rAAV2-TH and rAAV2-GCH1 strategy was explored further by utilizing a new generation of rAAV2 vectors where therapeutic levels of DOPA synthesis could be reached. At these levels of continuous DOPA synthesis, the animals did not only recover in drug-induced rotation tests but also showed improvements on a spontaneous motor test [55]. Notably, the magnitude of improvements were greater in animals with spared partial striatal DA innervation (in the order of 10-20% of normal) compared to animals with complete DA lesions (with <5% residual innervation), suggesting that the remaining DA terminals played an important role in mediating the therapeutic effects following the ectopic DOPA synthesis [55]. In line with latter data, Carlsson and colleagues recently showed that rAAV5-mediated DOPA delivery could reverse previously manifested L-DOPA induced dyskinesias in rats [56]. In this study, rats with intrastriatal 6-hydroxydopamine (6-OHDA) lesion with moderate-to-severe

behavioral impairments received daily pulsatile L-DOPA treatment until they were rendered stably dyskinetic. After striatal injection of rAAV5-TH and rAAV5-GCH1, the severity of the abnormal dyskinetic movements gradually decreased to about 15% of the initial scores at 12 weeks post injection [56].

We have recently complemented these encouraging results with proof that DA synthesized after this gene therapy approach reaches the post-synaptic receptors on striatal neurons in a physiological manner and that the therapeutic effects are correlated with normalization of the DA [57]. The study utilized the [^{11}C]-raclopride as a PET tracer to monitor the occupancy of the striatal DA receptors where the binding affinity (K_d) and the apparent maximum binding potential (B'_{\max}) could be quantitatively measured in a single imaging session. We found that complete DA lesion resulted in a decrease in K_d but did not affect the B'_{\max} . Importantly, the change in K_d could be reversed by the rAAV mediated continuous DOPA delivery, indicating that the ectopically synthesized DOPA was converted to DA and that it was biologically active in a physiological way at the DA synapses in the striatum [57].

Taken together, these data show that viral vector-mediated, continuous DOPA delivery is an attractive strategy for enzyme replacement in PD and should be pursued further with the ultimate goal being clinically tested for efficacy.

Modulation of basal ganglia circuitry

Neurosurgical intervention to reduce motor complications in PD has a long history and was the treatment of choice before the introduction of L-DOPA pharmacotherapy. In fact, already in 1947, the article reporting the first stereotactic surgery of the human brain described its application to modulate involuntary movements in patients

[58]. A couple of years later, lesion of the medial nucleus of the thalamus (thalamotomy) was shown to be effective on treating tremor [59]. During a quest for refined lesioning paradigms, it was found that lesions to the globus pallidus (pallidotomy) did not only have a positive effect on the tremor in patients with PD but also reduced fluctuations and dystonia [59, 60].

However, surgical lesions in the brain are irreversible and the effects difficult to predict. Thus, novel methods for site-specific inhibition of deep brain nuclei were explored. The most efficient solution was found to be an electrode, connected to a high frequency neurostimulator. This was first applied to the thalamus and was found to have a lower rate of complications than thalamotomy [61]. The mechanism of action for this intervention is not fully understood yet, but is thought to be via inhibition of neurons by depolarization in the volume influenced by the electrical probe. This technique is now, somewhat inaccurately, named DBS. DBS was later found to be efficient in other functional domains of the basal ganglia as well. Especially the internal segment of the globus pallidus (GPi) and the subthalamic nucleus (STN) gained much attention [60, 62]. The benefits of DBS in the STN in patients with PD include improvements in akinesia, rigidity and tremor to such extent that L-DOPA pharmacotherapy can be reduced which is also coupled with a reduction in L-DOPA induced dyskinesias [63].

Targeting STN using viral vectors for functional recovery in PD

The success of the DBS encouraged During and collaborators to use a molecular mechanism to inhibit the hyperactive glutamatergic drive originating from the STN in PD patients via gene therapy [64]. The enzymatic synthesis pathways for the excitatory glutamate (Glu) and the inhibitory GABA have many similarities. In fact,

GABA is synthesized directly from Glu by the glutamic acid decarboxylase (GAD) enzyme. Thus, over-expression of GAD in a glutamatergic neuron should lead to synthesis of GABA instead. Intriguingly, the vesicular transporter for Glu is highly specific and will not store GABA in vesicles [65, 66], therefore the storage and release of the pseudo-transmitter in the transduced cells is probably compromised. The initial experiments were conducted in intact and 6-OHDA lesioned rats, which received injections of rAAV2 vectors coding for GAD65 and GAD67 or the Green Fluorescent Protein (GFP) marker gene into the STN [67]. In these animals, GABA release was measured after activation of STN in the substantia nigra pars reticulata (SNr), a region innervated by neurons originating in the STN, which are normally glutamatergic. rAAV2 mediated over-expression of GAD65 lead to an increase in releasable GABA that was coupled to a decrease in amphetamine induced rotation and motor asymmetry in this unilateral lesion model. This study was later replicated in MPTP treated rhesus monkeys that received a unilateral injection of either rAAV2-GAD (mix of two vectors expressing either GAD 65 or GAD67) or rAAV2-GFP. As both the MPTP lesion and therapeutic manipulation was unilateral, the study focused on imaging outcomes and showed an increased metabolism in the ipsilateral motor cortex of the animals that ectopically expressed GAD enzymes compared to the control vector injected animals as assessed by [¹⁸F]-deoxyglucose ([¹⁸F]-DG) PET. There were no significant differences between the groups in behavioral scores obtained using the clinical rating scale [6].

The first clinical trial utilizing viral vector-mediated gene transfer in PD was conducted using this therapeutic strategy. An rAAV2 vectors coding for GAD65 and GAD 67 were infused unilaterally into the STN of twelve PD patients with UPDRS score of 30 or more in "off" state and/or complications of L-DOPA pharmacotherapy

(ClinicalTrials.gov registry number NCT00195143). The outcome of this safety trial was positive with no dropouts or patients lost to follow-up and no adverse events related to the gene therapy were reported [6]. The patients displayed improvements in motor UPDRS scores relative to baseline disability scores prior to gene therapy, predominantly on the side of the body that was contralateral to the treated side. The effect was seen first at 3 months after surgery and persisted up to 12 months when the report was published.

This clinical trial also utilized a novel imaging protocol that analyzed deviations from normal brain function and quantified restoration as therapeutic efficacy by measuring the modified cellular metabolism through imaging using [^{18}F]-DG PET and a complex analysis of linked changes in spatially distributed neural networks with related functions. With this approach, abnormal disease-related covariance pattern (PDRP) can be linked to motor manifestations of PD that differ from a PD-related cognitive pattern (PDCP) [68, 69]. In the rAAV2-GAD treated patients a significant reduction in thalamic metabolism and an associated increase in ipsilateral motor cortex metabolism was found at 12 months [5]. Based on these data, a Phase II randomized, multi-center, double blind, placebo control, safety/efficacy study has been launched and is currently ongoing (ClinicalTrials.gov registry number NCT00643890).

Disease modifying strategies

As PD is a progressive neurodegenerative disorder that affects some neuronal populations rather selectively, there has been an intense effort to find disease-modifying strategies to slow down or reverse the degenerative process. Although the patients would have typically lost about 50% of their nigral DA neurons and about

60-80% of striatal DA at the time of clinical diagnosis [63, 70, 71], the residual DAergic neurons constitute a significant substrate, on which therapies aiming at neuroprotection and repair might yield potent recovery from disease symptoms.

The glial derived neurotrophic factor (GDNF) was discovered in 1993 and has become the most studied molecule in the pursuit of neuroprotection in PD [72-74]. As a member of the transforming growth factor- β family, which also includes neurturin (NTN), neublastin/artemin and persephin, GDNF was first discovered in an *in vitro* assay because of its potent neurotrophic activity on the survival of midbrain DAergic neurons [74]. GDNF is expressed at high levels in the striatum during development and is maintained at low levels into adult hood. Knockout mouse models have severe deficiency in kidney development that lead to premature death of the animals [75]. In addition, GDNF expression in the adult intact or parkinsonian brain was found to be below detection limit [76]. Thus, initially the precise role of GDNF in the maintenance of DA neurons was not possible to demonstrate. More recently, however, a conditional knockout mouse model was utilized, where the GDNF expression was turned off in the adult mouse, leading to an about 60% reduction in nigral GDNF levels. This was sufficient to cause a more than 50% loss of nigral DA neurons within 7 months, supporting the view that GDNF was essential in the maintenance of the DA neurons in the adult life [77, 78].

The *in vivo* neuroprotective potential of GDNF has been studied in detail by administration of the purified recombinant protein either into intracerebroventricular (ICV) space or into the brain parenchyma. These studies showed good neuroprotective effect from single or repeated injections of GDNF or continuous infusion using pumps in both the rodent and primate models of PD [79-83]. Although

an extensive review of the data is beyond the scope of the present paper, it is sufficient to say that the magnitude of the neuroprotective effect seen after GDNF administration is critically dependent on the site of delivery, time and duration of treatment, as well as the mechanism of action the model employs to generate the DAergic lesion [80, 84-87].

GDNF infusion trials

The first clinical trial testing the efficacy of GDNF in PD patients was initiated already in 1996, a mere 3 years after the discovery of the molecule. Fifty subjects with moderate or advanced idiopathic PD were chosen for the study and received infusion of recombinant GDNF protein into the ICV space using mechanical pumps [88]. The study was conducted in a double blind manner and the patients on the active arm received single monthly bolus injections of 25 – 4000 µg GDNF into the lateral ventricle over 28 months, although only the first 8 months of the trial was blinded. The results showed not only that the patients did not improve in response to GDNF but also a number of side effects of the medication were observed [88]. These adverse events have in hindsight been attributed to GDNF stimulation of extra-striatal brain regions and the lack of therapeutic efficacy was thought to be due to the limited penetration of GDNF from the cerebrospinal fluid into the brain parenchyma. The latter was clearly demonstrated by post-mortem analysis in one of the patients from the study [89].

The failure of the first clinical study elucidated the need for alternative delivery paradigms that could achieve delivery at the expected site of action, i.e., in the putamen. In two subsequent open labeled clinical trials, L-DOPA responsive idiopathic PD patients received GDNF infusion via an intraparenchymal catheter

bilaterally into the postero-dorsal putamen [90, 91]. This time the results were more encouraging as both trials met the primary end-point with no serious adverse events. Furthermore, the patients experienced measurable benefits in motor function with 40% improvement in the motor sub-score of UPDRS at the one-year time point. In post-mortem histological examination of one patient, who died almost four years after onset of GDNF treatment, dense TH-immunoreactivity was found around the injection tract, probably due to sprouting of nigrostriatal fibers [92].

Based on these data, a multi-center, blinded, clinical trial testing the efficacy of intraputaminally recombinant GDNF protein infusion was initiated [93]. The results from this trial failed to confirm the preliminary observations of therapeutic efficacy reported in the open-label trials. Some patients displayed adverse events due to the GDNF infusion, including paraesthesias and headache. Although there was an increase in [^{18}F]-DOPA uptake around the infusion cannula in the posterior putamen of the treated patients, they actually displayed a tendency towards a worsening of the UPDRS scores. The discrepancies between the open label and blinded GDNF trials remain unclear, but differences in surgical protocols, selection of the patients in the trial, the infusion protocols and possible placebo effects have all been suggested to have contributed to the different outcomes between the previous open-labeled trials and the latter phase 2 trial [94].

Viral vector-mediated delivery of neurotrophic factors

Infusion of recombinant protein using intraparenchymal catheters was a good tool for proof-of-concept studies, however, for long-term, continuous delivery of GDNF other techniques had to be developed. Among those described in the literature, thus far,

viral vector-mediated gene transfer is undoubtedly the most powerful technique for this purpose. The first studies using this approach utilized a recombinant adenoviral vector to deliver the GDNF gene to the host brain. Injection into the rat SN prior to the 6-OHDA lesion showed that GDNF locally synthesized in the brain was able to protect DA neurons from the toxic insult [95]. However, the rescue of nigral neurons was not coupled to protection of striatal DA innervation and thus whether neuroprotection in the SN could be linked to functional rescue remained elusive. It was later found that both nigral and striatal injection delivery of GDNF resulted in protection of nigral DA neurons but only striatal GDNF over-expression was capable of sparing the DA fibers and decreasing the extent of the lesion [96]. First generation rAd vectors used in the initial studies were not ideal for *in vivo* gene delivery, neither in experimental settings nor in clinical applications, because they induced a severe inflammatory response in the brain. Thus, most of the studies aiming at gene therapy for GDNF, employed other recombinant viral vectors with better safety and efficacy profile, e.g., rAAV and lentiviral (rLV) vectors [97, 98]. (See [80, 87, 99-101] for in depth reviews of the topic.)

In studies using viral vectors to deliver GDNF, a number of important observations have been made: First, GDNF expressed in the striatum after gene transfer resulted in robust protein synthesis in the host brain. The GDNF molecule diffused very well within the brain parenchyma and was biologically active even at very low expression levels (representing only 2-3 fold increase over basal levels) [102]. The capacity to reach large areas in the target structure was in contrast to the outcome of the clinical trials utilizing protein infusion where poor tissue penetration was suggested to be one of the main reasons for the lack of efficacy [89, 93].

Another characteristic of GDNF over-expression is that it promotes intense sprouting of DA fibers when present in the host brain at the time of lesion [96, 103]. This might prove to be beneficial but may also be a reason for concern. On the one hand, if the sprouting occurs within the partially denervated striatum, the increased terminal density might compensate for the lost DA neurons and induce behavioral recovery or improved handling of peripheral L-DOPA. Ectopic sprouting e.g., in the globus pallidus (GP) or the SNr, on the other hand, might be less beneficial or even detrimental. Sprouting in the GP has been seen after striatal infusion of GDNF protein as well [104], but was more intense after viral vector-mediated GDNF gene transfer studies [102, 105-107]. This effect might be due to the efficient anterograde transport of the protein in the transduced striatal neurons projecting to this region.

Neurturin gene therapy

NTN was discovered in 1996, as the second member of the GDNF family of neurotrophic factors [108]. It was soon found to promote survival of midbrain DA neurons both *in vitro* and *in vivo* [109, 110]. NTN and GDNF share 42% sequence homology and both mediate action via formation of a receptor complex consisting of the tyrosine kinase RET [111] and a ligand-binding glycosyl-phosphatidylinositol (GPI)-linked subunit (GFR α). They differ in their affinity to bind GFR α subunits, GDNF preferentially binds to GFR α 1, whereas NTN binds to GFR α 2 (See [86] for a review of this subject). Nevertheless, NTN has been found to bind and promote action via GFR α 1 as well albeit at lower affinity [111].

Intracerebral delivery of recombinant NTN protein provided neuroprotective activity at about the same magnitude as GDNF in models of PD [112-114]. However, viral vector-mediated expression of NTN resulted in low synthesis

and poor neuroprotective efficacy *in vivo*. When synthesized in neurons, NTN is insufficiently processed and secreted as pro-NTN. Pro-NTN does not form signaling complexes with the Ret tyrosine kinase receptor and GFR α 1 or GFR α 2 and thus it does not have any biological activity [115]. By exchanging the signaling peptide to immunoglobulin heavy-chain signal peptide (IgSP) [115], or the pre-pro domain of nerve growth factor (NGF) [116], the secretion properties and biological activity of ectopically synthesized NTN could be regained. When these modified forms of NTN were delivered into the brain using rLV or rAAV2 vectors, the neuroprotective effects were very similar to GDNF with protection of midbrain neurons from striatal 6-OHDA lesion in the rat or intracarotid MPTP injection in the primate brain [115, 116].

In preparation for clinical trials, rAAV2-NTN was evaluated in the non-human primate MPTP model of PD, in aged primates and in 6-OHDA lesioned rats at a broad range of expression levels to show long-term efficacy and safety ([117-120]; see also ref. [101] for a recent review on this topic).

In the first (Phase I) trial, twelve PD patients received bilateral injection of rAAV2-NTN into the pre- and postcommisural putamen. (ClinicalTrials.gov registry number NCT00252850) [3]. The study was conducted in a dose-escalation design with the first six patients receiving 1.3×10^{11} vg of rAAV2-NTN and a second group of six patients a 4-fold higher dose. All patients received a total of 80 μ l of viral vector suspension stereotactically infused along four injection tracts and 2 deposits per track in each putamen, resulting in a total of 16 deposits. They were monitored for 12 months with extensive safety monitoring. The procedure was well tolerated, and the patients revealed no clinically significant adverse events at that timepoint [3]. The

efficacy of the therapy was assessed as a secondary end point. In several measures of motor function, the patients showed improvement at 1 year; e.g., a mean improvement of 14 points in the off-medication motor subscore of the UPDRS (36% mean increase) and a mean increase of 2.3 h (25% group mean increase) in on time without troublesome dyskinesia. Interestingly, both doses of the vector resulted in similar improvements. Non-motor symptoms were not significantly affected, nor were there any changes in [^{18}F]-DOPA uptake as assessed by PET imaging.

The follow-up study was designed as a double blind, multi-center phase II study testing the efficacy of rAAV2-NTN to reduce UPDRS motor “off” scores in PD patients (ClinicalTrials.gov registry number NCT00400634). This study included 58 PD patients with advanced disease. Two thirds of the patients were recruited to the active arm that received intraputamin rAAV2-NTN injections and the remaining third of the subjects were followed as placebo controls. Ceregene has recently announced that the study failed to reach its primary endpoint [121]. There were no significant differences between the active treatment and control group as both displayed a 7-point improvement in the UPDRS motor off score at 12 months. Although we need to wait for the publication of these data to have more accurate understanding reasons for the unexpected failure of the Phase II trial, it is possible to list a number of critical factors that might have contributed to the negative outcome. The first important point to note is that the primary endpoint of the study was symptomatic relief and not disease modification i.e., indication of neuroprotection, despite the fact that GDNF’s most extensively documented biological effect is neuroprotection. This somewhat different primary endpoint was probably preferred due to the experimental design and number of patients needed to unequivocally prove disease modification in PD patients. Furthermore, some data from the clinical infusion

trials have also indicated symptomatic relief rather than neuroprotective effects of GDNF; namely the observations after GDNF withdrawal. In one of the trials, the infusion solution was shifted to saline after 12 months, in a blinded manner, and the patients were monitored for another year. At 9 to 12 months after cessation of GDNF infusion, the UPDRS scores returned to baseline and the patients required increased pharmacotherapy [122].

A second important point is the fact that the pre-clinical efficacy of GDNF has been explored almost exclusively on animal models utilizing neurotoxin based lesions. This means that a critical assumption has been made; namely that neurotoxin based animal models reproduce the disease mechanism seen in PD patients. The closest example is probably the MPTP intoxication model in primates, as humans exposed to MPTP were shown to develop a parkinsonian syndrome [123, 124]. Nevertheless, whether or not the MPTP-induced parkinsonism cases share a common pathogenic mechanism with the idiopathic PD cases is not known. Needless to say, none of the toxin-based models are truly progressive or mimic the major pathological profiles seen in the parkinsonian brain, such as the formation of Lewy-bodies and neurites [125, 126]. In fact, Lo Bianco and collaborators utilized two rLV vectors to deliver alpha-synuclein and GDNF to the rodent SN. The rLV-GDNF vector was delivered 2 weeks prior to another rLV vector encoding the human alpha synuclein gene and the animals were followed for an additional 6 weeks. In this study they found no significant neuroprotective action of rLV-GDNF compared to the control vector (rLV-LacZ). Although this experiment did not provide a direct comparison between neuroprotective effects of GDNF in a toxic lesion model (e.g., 6-OHDA insult) and the alpha-synuclein overexpression, it raised concerns about the validity of toxin based models for development of neuroprotective strategies.

Thirdly, the Ceregene trial tested the efficacy of rAAV2-NTN in advanced PD patients. Although, this choice might have been made due to restrictions in patient populations that can be recruited to these experimental trials with invasive and irreversible procedures, it has a critical impact on the probability of the study to meet its presumed endpoints. Even for a symptomatic effect, GDNF could be efficacious only if there is a residual DAergic system present in the patient's brain that can be reached by the neurotrophic factor. It is this substrate that is likely to be the main factor affecting the outcome of the treatment. Thus, advanced PD cases are unlikely to be suitable candidates for this approach.

There is, however, an emerging opportunity for a second chance in the field. Amsterdam Molecular Therapeutics has recently announced that it had sealed an agreement with Amgen to license GDNF for rAAV mediated gene therapy in PD [127]. Provided that a carefully planned study addressing potential shortcomings of the previous trial can be developed, we are optimistic that successful results can be obtained with neurotrophic factors in PD.

Concluding remarks

The recent progress we have seen in gene therapy for PD illustrates the rapid advancement of viral vectors as clinical tools for therapy in the brain. As we tried to elucidate, the currently pursued strategies are based on several years of research in fields as diverse as neurology, pharmacology, molecular biology, virology and imaging. We think that it is only with a thorough understanding of these fields that novel, safe and efficient gene therapy strategies can become clinically applicable treatments. It has also become clear that there will probably be a need for multiple alternative therapeutic strategies that act via different mechanisms, to provide optimal

treatment to each PD patient who might be suffering from different causes or severity of disease. In figure 2 we provide a summary of our views and illustrate the different determining points, how different interpretations of clinical and experimental data can be used to leverage different approaches to treatment. In the next few years, we will see the completion the first phase I and phase II trials testing recombinant viral vectors as therapeutic tools in the clinic. Whether or not the individual trials meet their primary end-points, the results from these initial trials are going to give us very precious information in the refinement of this powerful tool and a substrate on which better therapeutic products can be designed. It is our strong belief that in vivo gene transfer techniques will eventually become an important addition to the therapies offered to PD patients in the future.

Figure legends

Figure 1. Schematic drawings of viral vector-mediated enzyme replacement strategies in the parkinsonian brain. (A) *Pro-drug approach for enhanced DA synthesis.* rAAV mediated gene transfer of AADC results in expression of the enzyme at high levels in striatal neurons from an episomal plasmid. L-DOPA from oral pharmacotherapy enters the brain over the blood-brain barrier. It is taken up by the transduced striatal neuron and converted by the ectopic AADC to DA that probably accumulates in the cytoplasm and diffuses towards the post-synaptic DA receptors providing symptomatic relief. (B) *Dopamine replacement.* The multi-cistronic EIAV vector infects the striatal neuron where the genes are integrated in the host genome. As the vector carries all three genes required for DA synthesis (TH, GCH1 and AADC), dietary tyrosine can be used to synthesize DA within the striatal neuron at a constant rate. This DA then exits the neuron and reaches the DA receptors in the same way as in panel A. (C) *Continuous DOPA delivery strategy.* rAAV mediated gene transfer of TH and GCH1 results in transduced striatal neurons that can synthesize DOPA from dietary Tyrosine at a constant rate. However, as the cells do not possess the decarboxylase activity, the DOPA exits the cell and ends up in spared DA fibers or Serotonergic terminals. There it is converted by endogenous AADC enzyme into DA and stored in vesicles by the VMAT2 transporter. The ectopically derived DA (in blue) therefore competes with endogenous 5-HT or DA (in red) for storage and release.

Figure 2. Conceptual flowchart describing a rational decision tree for selection of optimal gene therapy strategy and a short list of pros and cons for the respective approach.

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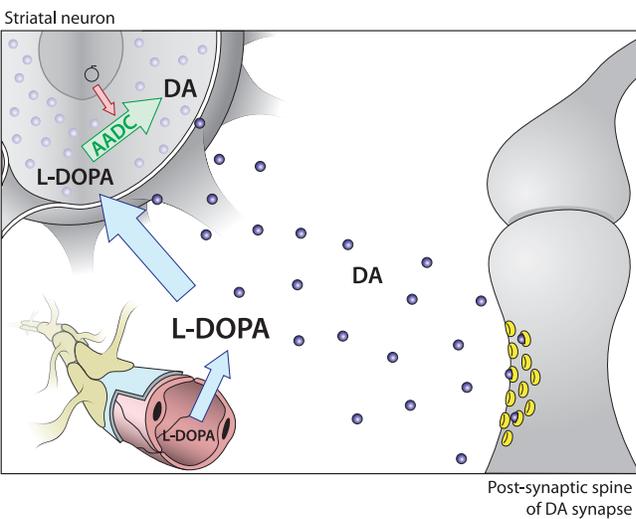
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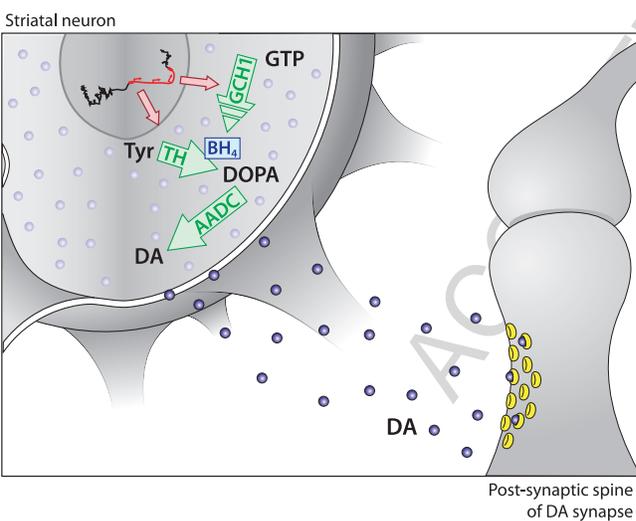
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ACCEPTED MANUSCRIPT

A. Pro-drug approach for enhanced DA synthesis



B. Dopamine replacement



C. Continuous DOPA delivery strategy

