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Review

Gene therapy for Parkinson's disease: Disease modification by GDNF family of ligands

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ABSTRACT

Gene transfer is a promising drug delivery method of advanced therapeutic entities for Parkinson's disease. One advantage over conventional therapies, such as peripheral delivery of the dopamine pre-cursor L-DOPA, is site-specific expression of proteins with regenerative, disease-modifying and potentially neuroprotective capacity. Several clinical trials have been performed to test the capacity of glial-cell line derived neurotrophic factor and neurturin to rescue degenerating dopaminergic neurons in the substantia nigra and their axon terminals in the striatum by delivery of these neurotrophic factors either as purified protein or by means of viral vector mediated gene delivery to the brain. Although gene therapy approaches tested so far have been shown to be safe, none met their primary endpoints in phase II clinical trials designed and powered to test the efficacy of the intervention. Within the scope of this review we aim to describe the state-of-the-art in the field, how different technical parameters were translated from pre-clinical studies in non-human primates to clinical trials, and what these trials taught us regarding important factors that may pave the way to the success of gene therapy for the treatment of Parkinson's disease.

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the decrease in dopamine (DA) input to the striatum resulting in a debilitating loss of motor control and the symptoms of tremor, rigidity, akinesia and postural imbalance. Another hallmark of the disease is the accumulation of protein inclusions, Lewy bodies, in several nuclei in the brain including, but not limited to, the DA neurons in substantia nigra pars compacta (SN). For the majority of PD patients the etiology is unknown although possible risk factors include old age, toxin exposures and genetic factors (Alves et al., 2013; Wirdefeldt et al., 2011). Today, a causal genetic mutation can be determined in about 10% of cases.

Symptomatic treatment of PD is focused on supporting the DA tone and function in the striatum - the main target nuclei for DA projection neurons in SN. The precursor of DA, L-3,4-dihydroxyphenylalanine (L-DOPA), is used for symptomatic relief since DA has a very short half-life, causes hemodynamic effects when given peripherally and is unable to cross the blood–brain barrier (BBB) due to its polarity. Endogenously synthesized L-DOPA normally derives from the dietary amino acid tyrosine, a reaction catalyzed by the tyrosine hydroxylase (TH) enzyme. The enzymatic activity of TH is dependent on the presence of the co-factor 5,6,7,8-tetrahydro-L-biopterin (BH4). L-DOPA is then further converted

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into DA by L-aromatic amino acid decarboxylase (AADC). In the normal intact brain AADC activity is far greater than TH and is found in multiple cell compartments other than DA neurons, e.g., serotonergic cells, thus the rate limiting step in synthesis of DA is the activity of the TH enzyme.

L-DOPA medication is given in combination with benserazide or carbidopa to inhibit the peripheral AADC activity and minimize the conversion of L-DOPA precursor to DA outside the brain. Although the therapeutic benefit of L-DOPA is striking in the beginning of treatment, troubling side effects appear in the majority of patients after some years. These side effects involve involuntary movements, so called dyskinesias, that are believed to emerge when the therapeutic window becomes narrower and the dose of L-DOPA needed for an anti-parkinsonian effect (typically within the motor domain) is increased due to increasing loss of DA neurons. Intermittent availability of L-DOPA after oral administration and fluctuations in drug levels in the brain cause the patients to experience rapid shifts from parkinsonian to dyskinetic state and vice versa. Sustained release formulations with L-DOPA have been explored to stabilize plasma levels of L-DOPA but the therapeutic benefit is less potent. Other attempts to increase the therapeutic benefit of L-DOPA aim at preventing the degradation of DA in the synapse by inhibition of the monoamine oxidase (MAO) and the catechol-o-methyl transferase inhibitor (COMT). Alternatives to L-DOPA include DA agonists that directly target the post-synaptic DA receptors. These agonists are often used to allow delayed initiation of L-DOPA therapy and thereby increase the total time of efficacious anti-parkinsonian treatment.

The concept of drug delivery by gene transfer, i.e. gene therapy, has many attractive attributes in the treatment of PD and other neurodegenerative diseases. First, many viral vectors are able to efficiently transduce brain cells resulting in long-term expression of the transgene. Secondly, intracerebral injection of vectors allows the expression of transgenes in specific brain areas, which reduces the off-target effects that are often associated with systemic drug administration. This specificity can be further improved by using specific vector serotypes and promoters to drive the expression of the therapeutic gene in restricted cell groups in the target area.

The adeno-associated viral (AAV) vector is the most frequently used vector for neurological disorders and there are currently 7 ongoing clinical trials for PD. AAVs belong to the *Parvoviridae* family and are single-stranded DNA viruses, where the recombinant vector is non-integrating and requires a helper virus to generate new virions. When the virulent genes are removed, leaving only the inverted terminal repeats (ITR), the AAV vectors are not considered to be pathogenic in humans - especially since modern production methods do not require co-infection with wild type helper virus (2016). AAV vectors are very suitable for CNS gene therapy since they transduce neurons with high efficiency. This can in particular be illustrated in cases where the therapeutic transgene is a cytoplasmic protein allowing the identification of transduced cells in the brain (Fig. 1). Neuronal transduction results in long-term transgenic expression and MPTP lesioned monkeys have shown persistent transgene expression for up to 8 years post-injection, which has

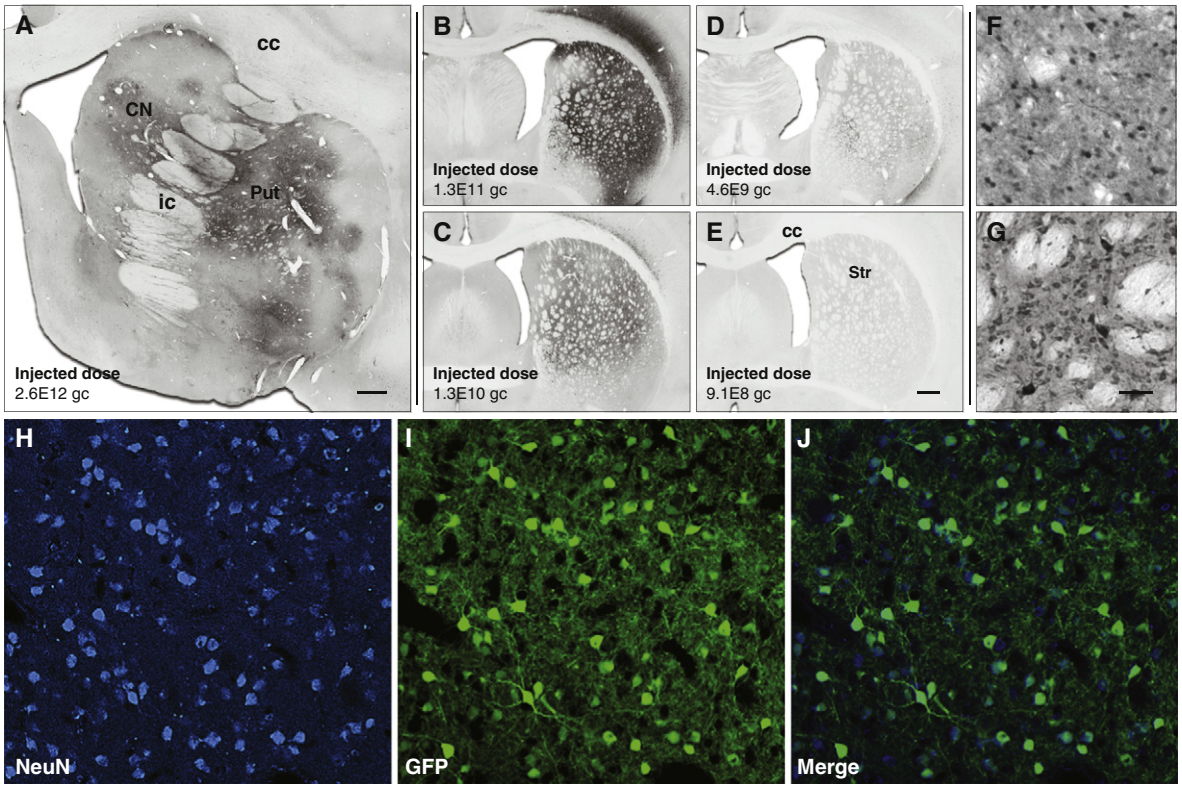


Fig. 1. Immunohistochemical staining for AAV vector-derived transgenic GTP cyclohydrolase 1 (GCH1) in rhesus monkey (A,F,H–J) or rat (B–E,G). GCH1 is the rate-limiting enzyme in the synthesis of the co-factor tetrahydrobiopterin - essential for efficient function of tyrosine hydroxylase (TH) and dopamine (DA) production. It is shown here as an example of a therapeutic transgene with cytoplasmic localization and thus different from neurotrophic factors reports the identify of transduced cells in the target region. All animals were injected with the same vector construct, encoding human GCH1 and TH, in the caudate and putamen. Only human GCH1, and not endogenous forms of the protein, was recognized by the antibody. In the rhesus monkey (A), injections were made at two sites in the caudate nucleus (10 + 5 μl) and at three sites in the putamen (10 + 10 + 5 μl) making a total of 40 μl injected per hemisphere. Injections in rodents were made at two sites (in total 5 μl) within the dorsal striatum. Injections of 1.3E11 gc resulted in wide-spread transduction including extrastriatal areas e.g. globus pallidus and overlying cortex (B). Decreasing the dose 10-fold resulted in similar transduction pattern in the striatum but much more limited spread to adjacent regions (C). Decreasing the dose to 4.6E9 and 9.1E8 gc resulted in sparse transduction with only few immunoreactive cell bodies. High-power images are taken from the transduced putamen in monkey (F) and from the dorsal striatum in the rat (G). Panels H–J are confocal images stained with NeuN (H) and GFP (I) taken from another monkey. Merged channel is shown in J and illustrates that the transduced cells are essentially all neuronal phenotype. Scalebar in A represent 1 mm, E represent 0.5 mm, G represent 50 μm. cc: corpus callusum, CN: caudate nucleus, gc: genomic copies, ic: internal capsule, Put: putamen, Str: striatum.
Panels modified from Cederfjäll et al. Scientific Reports, 2013.

been shown by maintained response to L-DOPA and increased signal with 6-[(18)F]fluoro-meta-tyrosine (FMT) positron emission tomography (PET) compared to their pretreated state (Bankiewicz et al., 2006; Hadaczek et al., 2010). On the other hand, efficient transduction of non-neuronal cells in the brain require the use of modified vector capsids and/or promoter elements that restrict the expression to these cells (Drinkut et al., 2012; Gray et al., 2011b). Several steps in the intracellular processing of the AAV particles have been reported to be rate limiting for transduction of the host cells. Although not mutually exclusive these factors are thought to include proteasomal degradation of the capsids, entry of the particles to the nucleus and complementary second strand synthesis (Ferrari et al., 1996; Grimm et al., 2006; Zhong et al., 2008). These limitations can be avoided by e.g., the use of self-complimentary AAV (scAAV) vectors, modification of capsid residues that are targeted during the degradation, which would decrease the latency process as well as the efficacy of the transduction several folds. Notably, one limitation of scAAV vectors is the reduced loading capacity of the virus' native 4.9 kb to approximately 2.2 kb (Gray et al., 2011a; Hollis et al., 2008; McCarty et al., 2003; McCarty et al., 2001). Although AAV2 is the most commonly used serotype, more recently several other serotypes have been investigated to optimize transduction efficiency and spread in different species (Dodiya et al., 2009; Kornum et al., 2010; Markakis et al., 2010). Dodiya and colleagues studied which AAV vector serotype would most efficiently express green fluorescent protein (GFP) in the non-human primate (NHP) brain as reference for clinical translation (Dodiya et al., 2009). GFP expression was compared after intrastriatal delivery of AAV1, AAV5 and AAV8 in six healthy adult cynomolgus macaques. AAV1 and AAV5 transduced significantly more cells and resulted in a larger spread than AAV8. To date, AAV2 is the only serotype used in clinical trials for PD. Importantly, however, other serotypes have already entered into clinical testing within the context of other neurological conditions and may prove to be better candidates also for PD (ClinicalTrials.gov Identifier: NCT01474343, NCT01299727).

2. Clinical translation of neurotrophic factor gene therapy strategies for Parkinson's disease

To date, two approaches using AAV gene therapy to deliver neurotrophic factors for PD advanced to clinical trials (Table 1). Although the fundamental principle for testing neurotrophic factors in PD is based on the principle that it would be possible to slow down the disease progression and regenerate damaged neurons, neither the trials that have been completed recently, nor the trial that is ongoing at time of writing this review will test this hypothesis. Effectively, they have been designed to explore whether patients in late stage disease with complications will respond to the treatment, most likely via a mechanism that would have a significant contribution through symptomatic relief rather than neuroprotection.

Glial-cell-derived neurotrophic factor (GDNF) and neurturin (NRTN) are two neurotrophic factors that have been extensively studied in experimental animal studies for their neuroprotective potential in PD. Both GDNF and NRTN function via a signaling complex constituted by RET tyrosine kinase, and the GDNF family receptor α (GFRα) 1 or 2 respectively. Initial in vitro studies showed the capacity of GDNF to promote cell survival in mesencephalic cell cultures (Clarkson et al., 1997; Lin et al., 1993; Meyer et al., 2000). GDNF was at first delivered by intraventricular infusion of the recombinant protein in solution and encouraging results in neurotoxic models of PD lead to the initiation of several clinical trials (Beck et al., 1995; Gash et al., 1996; Kirik et al., 2000; LeWitt et al., 2011; Miyoshi et al., 1997; Tomac et al., 1995). In a phase I–II, randomized, double-blinded clinical trial, fifty PD patients received monthly bolus injections of GDNF (under the name Liatermin or r-metHuGDNF) via an implanted intracerebroventricular catheter. The outcome was negative as GDNF failed to improve the patients' scores in the Unified PD Rating Scale (UPDRS) and many developed side effects like nausea, paresthesia and weight loss (Nutt et al., 2003). The lack of efficacy was believed to be due to an inadequate delivery method as the post-mortem analysis in one of the cases showed no detectable

Table 1
Neurturin: Summary of pre-clinical and clinical studies. All studies utilized AAV serotype 2 where neurturin was under a CAG promoter.

Study type	Disease stage	Subjects to treat	Side	Target nucleus	Total volume injected per hemisphere (μl)	Total genomic copies injected per hemisphere	Outcome	Reference	ClinicalTrials.gov identifier
Pre-clinical	*Macaca mulatta* (young, MPTP injected)	n = 5	Unilateral	Pu Ca SN	30 45 10	1.7E11	*Neuroprotection *88% reduction in UPDRS scores *Expression of NRTN in caudate-putamen and SN	Kordower et al. (2006)	
	Macaca mulatta (aged)	n = 3	Unilateral	Pu Ca	90 60	3.0E11	*Increased FMT uptake *Expression of NRTN in caudate-putamen	Herzog et al. (2007)	
Phase Ia	H&Y 3–4, >30 UPDRS OFF	Low dose n = 6 High dose n = 6	Bilateral	Pu	40 40	6.5E10 2.7E11	*Safety *Tolerability	Marks et al. (2008)	NCT00252850
Phase Ib	H&Y 3–4, >30 UPDRS OFF	Low dose n = 3 High dose n = 3	Bilateral	Pu SN Pu SN	40 30 150 30	4.7E11 1.2E12	*Safety *Tolerability	Bartus et al. (2013)	NCT00985517
Phase IIa	>30 UPDRS OFF (part III)	n = 38	Bilateral	Pu	40	2.7E11	*37 patients were included in primary analysis. Primary baseline to 12 months: UPDRS part 3 score in OFF state AAV-NRTN −7.21 + −1.56 compared with Sham surgery −6.91 + −2.12	Marks et al. (2010)	NCT00400634
Phase IIb	>30 UPDRS OFF (part III)	n = 24	Bilateral	Pu SN	150 30	1.2E12	*Did not meet primary endpoint of significant reduction in UPDRS OFF score *CERE-120 resulted in significant improved secondary endpoint of self-reported daily diaries (Diary-OFF score)	Warren Olanow et al. (2015)	NCT00985517

GDNF protein in the target region of the brain (Kordower et al., 1999). Indeed, targeting the putamen directly with continuous infusion pumps in a second trial resulted in less side effects and improved the off-state UPDRS scores by 48% in 5 patients at 12 months albeit in an open-label study design (Gill et al., 2003; Love et al., 2005; Patel et al., 2005). Continuous administration at varying doses of GDNF not only improved the clinical outcome but also increased the uptake of [(18)F]fluoro-DOPA (Fluorodopa) around the GDNF target site in the putamen and the correlated SN region on PET scans (Love et al., 2005). Moreover, an increased signal was observed in anatomical T2-weighted images that corresponded to the tip of the catheter and reflected infused volumes. The extension of this trial to a further 12 months showed a sustained benefit of GDNF continuous delivery on off-medication UPDRS scores in all patients without side effects (Patel et al., 2005).

Another open label study on 10 advanced PD patients where unilateral intraputaminal infusions using a multiported catheter and a precise delivery schedule (as opposed to continuous) were used. This study obtained the same results as Gill et al. over 12 months and importantly the improvement in off-medication UPRDS score was lost once GDNF infusion was discontinued. As a follow up from these observations, 34 patients with advanced PD were recruited in a placebo-controlled double-blind phase II clinical trial (Lang et al., 2006). Patients in the treatment group received bilateral intra-putaminal infusion of 15 μg recombinant GDNF protein per hemisphere - the same dose tested in the pilot study - and the primary endpoint was defined as change in UPDRS score off medication at 6 months. The trial did not meet its pre-defined primary end point as there was no improvement in UPDRS scores above that of the control arm even if secondary outcome measures such as Fluorodopa PET scans revealed increased uptake in GDNF-treated versus placebo-treated patients (Lang et al., 2006). These negative functional outcomes combined and safety issues that were raised when 10% of patients were found to carry antibodies against the recombinant GDNF protein, the development track for GDNF protein delivery was halted in 2004. Of note, the recombinant protein used in the above studies were produced using bacteria as the producer organism rather than mammalian cells and this choice has significant implications on the biological activity of the resulting recombinant protein product due to differences, among others, in glycosylation patterns that are thought to influence the stability and formation of disulphide bridges affecting the folding and therefore the activity of the protein (Hoane et al., 2000; Piccinini et al., 2013).

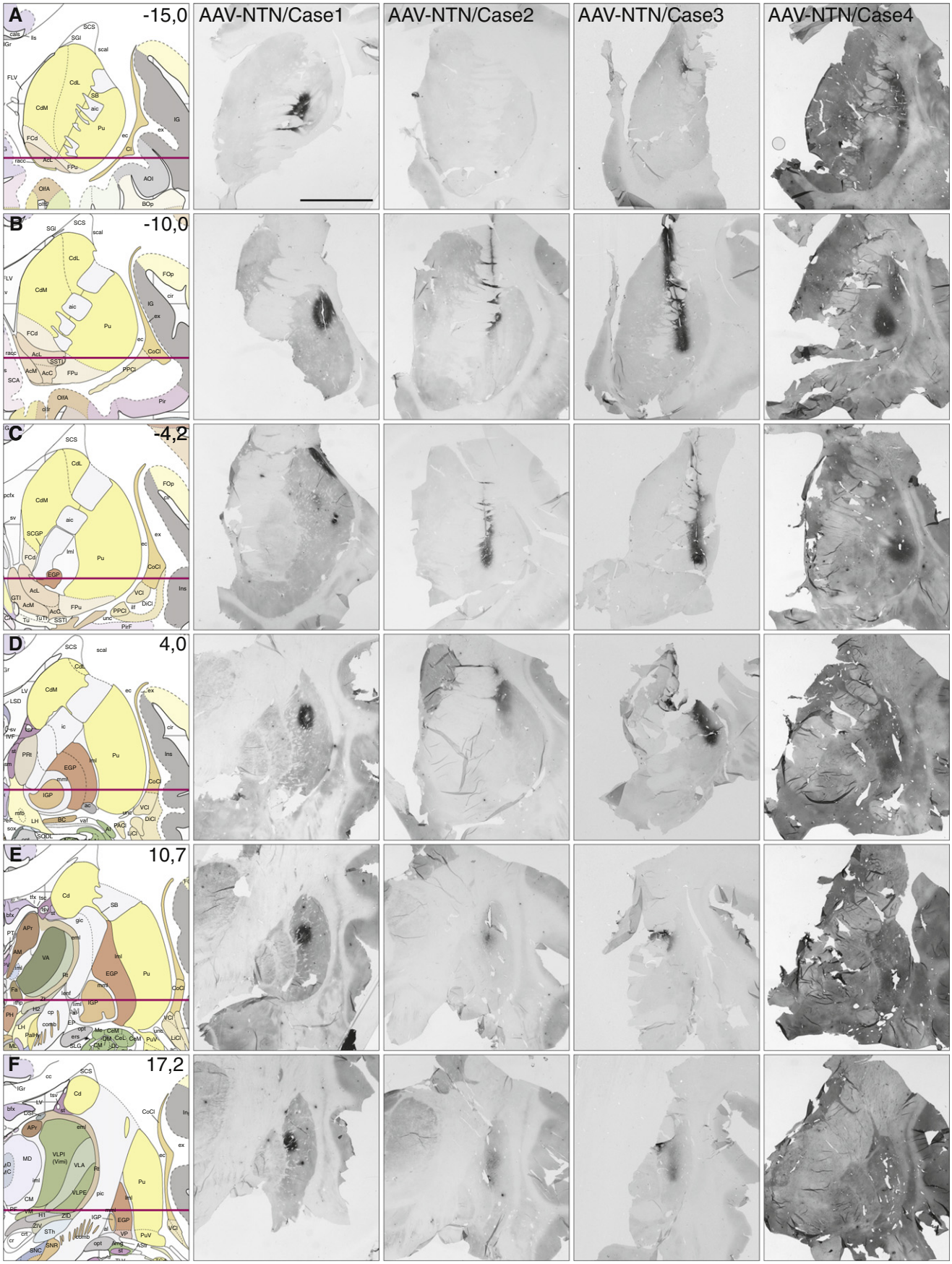
Gene therapy was pursued as an alternative way to deliver GDNF to the brain by transducing neurons with vectors encoding the transgene. Kordower et al. administrated a lentiviral vector expressing GDNF into the striatum and SN of non-lesioned aged or young adult MPTP-treated NHP. Three months later GDNF immunoreactivity could be detected in all vector-treated animals in the injected striatum and SN. In the aged NHP model, the animals displayed increased Fluorodopa uptake in the treated hemisphere compared to baseline Fluorodopa PET scans that was further confirmed by post mortem TH striatal immunostaining (Kordower et al., 1999; Kordower et al., 2000). Similar results were obtained in the MPTP lesion model where the GDNF-expressing lentiviral vector was administrated 1 week prior to a unilateral lesion. Nigrostriatal degeneration was completely prevented and flourodopa PET signal was increased in the treated striatum compared to β-Gal injected controls. Moreover, two further NHP sacrificed at 8 months post-infection showed neuroprotective effects on the nigrostriatal neurons, improved motor function and transgene expression in the absence of an immune response (Kordower et al., 1999; Kordower et al., 2000).

A large number of studies were carried out with AAV2-GDNF in both rodent models of PD, aged as well as MPTP-lesioned NHP models. Results from the rodent studies suggested GDNF had a protective effect when injected in the striatum and SN either before or after the neurotoxin lesion, however, only striatal transduction resulted in behavioral recovery (Kirik et al., 2000). In the 6-OHDA marmoset NHP model, unilateral injection of AAV2-GDNF into the SN four weeks before lesion reduced the loss of DA neurons by 40%, and up to 84% in a follow up study, compared to the non-lesioned hemisphere (Eslamboli et al., 2003; Eslamboli et al., 2005a). Furthermore, studies with AAV2-GDNF in both aged and unilaterally lesioned MPTP NHPs showed a PET increase in FMT uptake that was correlated to an increase in GDNF expression in the putamen and resulted in locomotor recovery or clinical score improvement respectively (Eberling et al., 2009; Johnston et al., 2009; Kells et al., 2010). In particular, Kells et al. injected NHP 3–6 months after a unilateral MPTP-lesion to study the effects of GDNF in NHP with extensive DA denervation and a stable parkinsonian phenotype. Vector-treated NHP showed a gradual improvement in clinical score up to 18-months after receiving AAV2-GDNF, which was associated with a PET increase in FMT uptake in the putamen (Kells et al., 2010). Interestingly, there is currently an ongoing open label phase I clinical trial with AAV2-GDNF funded by the NIH, which includes advanced PD patients in a dose-escalation, safety and tolerability study design (ClinicalTrials.gov Identifier: NCT01621581).

NTRN has been shown to enhance survival of DA neurons in vitro and in vivo in the 6-OHDA lesion model in the rat following protein infusion or intraparenchymal delivery of engineered cells (Grondin et al., 2008; Horger et al., 1998; Liu et al., 2007a; Liu et al., 2007b; Oiwa et al., 2002; Rosenblad et al., 1999; Ye et al., 2007). Moreover, direct comparison of the functional effects of GDNF and NTRN in these models has demonstrated similar efficacy, at least when delivered directly in the parenchyma. CERE-120 is a clinical product developed by Ceregene Inc., which consists of an AAV2 vector encoding a modified cDNA for NRTN, where the pre-pro domain of the protein has been replaced with the human nerve growth factor counterpart. Its efficacy and safety after intraparenchymal delivery was demonstrated in several rodent and NHP models of PD where functional recovery and DA neuron preservation was observed in the absence of adverse effects (Gasmi et al., 2007; Herzog et al., 2009; Herzog et al., 2008; Herzog et al., 2007; Horger et al., 1998; Kordower et al., 2006; Kotzbauer et al., 1996). In particular, a study conducted by Kordower and colleagues in aged NTRN-treated NHP showed an ipsilateral increase in Fluorodopa PET signal and a significant increase in TH immunoreactivity in the striatum up to 8 months post-infection (Herzog et al., 2007).

After an initial open label safety trial documenting the safety of the approach on 12 PD patients (Marks et al., 2008) a controlled double blind phase II trial was performed to further confirm its safety and to assess its therapeutic efficacy. Fifty-eight PD patients were enrolled where 38 received the CERE-120 and 20 subjects were followed as sham-operated controls. The safety profile of the vector was again favorable; however, the study did not reach its primary endpoint at the 12-month assessment (Marks et al., 2010). Post-mortem brain tissue

Fig. 2. Human post mortem sections from the 4 cases that have come to autopsy in the AAV-NRTN trial. Six rostrocaudal levels covering the caudate nucleus and putamen are shown. Left column are illustrations from the human brain atlas representing the levels shown in the panels to the right (A–F). The precise position of each level in the atlas is depicted on the right upper corner (in mm based on a coordinate system with zero point located in the center of AC commissure, AC-PC line based space). The red line indicates the axial plane running through AC center. Positive y-values of the slices indicate posterior position to AC center. AAV-NRTN/Case 1 and Case 2 were patients that died 1.5 and 3 months after the intervention for unrelated reasons. Brains were collected with 13 and 6 hours post-mortem delay, respectively. Cases 3 and 4 were autopsied at 4 and 4.3 years after injection of the vectors with post-mortem delays of 4.5 and 25 hours, respectively. Case 3 has a pathologically confirmed MSA-P diagnosis. Striatal atrophy is visible in this case. The human atlas panels were reproduced with permission from Elsevier and kindly provided by Milan Majtanik (University of Düsseldorf, Germany). The human post-mortem specimens are a courtesy of Drs Jeffrey Kordower and Yaping Chu (Rush University, Chicago), while the photographs were taken and figure assembled by DK. Scale bar in Case 1 represents 1 cm and is applicable to panels from all cases. Note that the scaling of the human atlas images does not precisely match the post mortem specimens and are thus only for purposes of orientation to relevant nuclei in the stained specimens.


A
-15,0
AAV-NTN/Case1
AAV-NTN/Case2
AAV-NTN/Case3
AAV-NTN/Case4
B
-10,0
C
-4,2
D
4,0
E
10,7
F
17,2

from two CERE-120 injected patients that had died from unrelated causes revealed that the putamen was successfully transduced. However in contrast to NHP, where a lower dose CERE-120 resulted in transduction of SN neurons, only few NRTN immunoreactive cells were observed in the SN from the patients suggested that there may be differences in protein transport in NHPs and humans with PD (Bartus et al., 2011b) (see also additional data shown here in Fig. 2). In fact, the authors argued that the lack of therapeutic efficacy at the pre-defined end-point of 12-month follow-up in this trial was due to impaired retrograde transport in nigrostriatal neurons in advanced PD patients. Furthermore, they suggested that this phenomenon could explain the late clinical improvement observed at 15 and 18 months after treatment compared to sham controls. These observations formed the basis for conducting additional pre-clinical studies where SN was targeted in combination with the putamen (Bartus et al., 2011a; Herzog et al., 2013; Su et al., 2009). The new injection protocol consisted of three deposits along three needle trajectories in the putamen and two deposits along a single needle trajectory in SN. In addition, the dose of CERE-120 was increased 4-fold in the putaminal site, whereas the same vector dose used in the previous phase IIb clinical trial was used in SN. In this new phase I trial, 6 patients were injected using the new injection protocol - three received 8E11 vg/patient and the remaining three received 3.1E11 vg/patient (Bartus et al., 2013). At 24 months, the new injection paradigm and dosing of CERE-120 met the safety endpoint. Based on these results a placebo-controlled double blind phase II trial was initiated with 51 patients with advanced PD that were divided into treatment ($n = 24$) and sham ($n = 27$) surgery groups. Patients in the treatment group received bilateral injections of 2.0E11 and 1.0E12 vector genomes in the SN and putamen, respectively (Warren Olanow et al., 2015). The primary endpoint in this trial was defined as the change from baseline to last blinded assessment (at 15 months) in UPDRS Part 3 scores performed off-medication. The longer evaluation period was chosen to pick up possible therapeutic effects that were observed in the earlier phase II trial at 12 months. Forty-seven patients completed the study and at the 15-month assessment time point no significant difference in UPDRS score was observed between the two groups. Also, no secondary endpoints reached significant difference between the groups, which included total UPDRS, UPDRS on medication and the "Activities of daily living" scale.

3. Methodological considerations and scale up

The gene therapy clinical trials that have been performed in this area highlight the large gap between positive pre-clinical data and successful clinical translation beyond accomplishing safety in humans. All major gene therapy clinical trials in PD have been supported by extensive pre-clinical data in at least two different species and disease models and all have shown an excellent safety profile and clear documentation of efficacy. Yet, these encouraging results did not translate into successful efficacy documentation in clinical trials. One plausible reason for the failure in translation of the neurotrophic factor delivery strategies in PD could be related to species differences between rodent, NHP and humans including brain size, anatomical organization and immunological aspects. Moreover, as clinical trials rely on the data in available animal models, this relationship presents itself as a potential caveat and a weakness in decision processes in not only the selection of the therapeutic entity to be tested but also the anticipated efficacy in humans.

Animal models of PD have known limitations in recapitulating the critical aspects of the disease phenomenon in humans. The use of acute toxin-induced models to explore the chronic degeneration that characterizes PD in patients is a limitation in itself. Not only the mechanism of neurodegeneration induced by these lesions is not a replica of the human disease, but also the progression is too rapid leaving little or no room for compensatory mechanisms to both act and wear-off over time. The importance of timing of the intervention was recently highlighted in a study by Fu and colleagues on a gene therapy approach for the lysosomal storage disease MPS IIIA. The authors used intravenous injections of a scAAV9 expressing *N*-sulfoglucosamine sulfohydrolase as the therapeutic gene in a mouse model of the disease and performed the intervention between 1 and 9 months of age. Interestingly, although the efficiency of the gene expression was not altered, the latest time-point tested did not increase life-span of the animals (Fu et al., 2016).

Models of PD are rarely established in aged animals, instead young adult animals are routinely used as experimental subjects. It is difficult to compare the status of the brain in young adult animals in terms of metabolism and function including the compensatory capabilities with that of PD patients at any stage of the disease. An acute lesion to the DA system in a young and otherwise healthy animal probably fails to reflect the lack of plasticity, inefficient metabolism and connectivity deficits that underlie the ongoing degenerative process in aged PD patients and therefore gives limited clues on the most appropriate time and mode of intervention. Indeed, by definition, animal models tend to reproduce a single well-known aspect of one (and rarely multiple) pathologenic process(es) that most likely neglects co-morbidity factors that could certainly affect treatment outcomes. Furthermore, none of the animal models employed in the pre-clinical efficacy testing of the gene therapy strategies form any alpha-synuclein immunopositive inclusions, which is an important hallmark of PD pathology in humans. This discrepancy may be important for the correct interpretation of the outcome in the pre-clinical studies and deserves further attention.

Recent studies have suggested that toxic oligomeric forms of alpha-synuclein play a role in the pathogenic cascade in PD possibly involving a mechanism of spreading between cells (Brahic et al., 2016; Freundt et al., 2012; Hansen et al., 2011; Kovacs et al., 2014; Rey et al., 2013). According to the Braak staging of PD neuropathology, alpha-synuclein immunopositive Lewy bodies and neurites begin in the dorsal motor nucleus of the glossopharyngeal and vagal nerves and the anterior olfactory nucleus and spread in the rostral direction (Braak et al., 2003; McCann et al., 2015). Of note, many recent studies have shown significant SN cell loss in the absence of SN alpha-synuclein deposition in those with these early Braak stages of PD (Dijkstra et al., 2014; Frigerio et al., 2011; Iacono et al., 2015; Milber et al., 2012). These findings suggest that, in the earliest stages, PD has quite different effects on these different neuronal populations, a concept that has implications for what brain areas to target with growth factor delivery designed to maintain existing neuronal health. Overall, experiments in animal models manifesting regionally selective versus widespread synucleinopathy should test if and how neurotrophic factor delivery can effect these separable pathologies to provide further insight on how translation of the treatment strategy from animal models to the clinic can be successfully accomplished. Needless to say, a robust intervention will have to be based on a therapeutic strategy targeting the core of the disease to be effective in a highly heterogeneous patient population with regards to disease onset, progression and degree of pathology, otherwise, the treatment will have to be adapted and developed for a small segment of the patients. At present, we do not know if neurotrophic factor signaling in the diseased neurons act at this desired level in the brain.

In the experiments conducted using neurotoxic PD animal models, neurotrophic factor delivery has been targeted to the striatum with an intended effect on the nigro-striatal pathway. Importantly, however, the transduced neurons themselves and other neurons in the striatum that are exposed to the neurotrophic factor released from the transduced neurons are also responsive to these factors. It is thus possible that growth factors delivered in this manner exert effects on the post-synaptic striatal neurons as well and this aspect of their biological action is not fully accounted for. In fact, striatal neurons known to be severely affected in the neurodegenerative Huntington's disease (HD) have been reported to respond to GDNF and NRTN. The first studies were performed in neurotoxic models of HD using quinolinic acid or 3-nitropropionic acid to produce striatal pathology. GDNF or NRTN

delivered by transplanted cells, intraventricular delivery or AAV vectors was found to have protective effects on striatal neurons (Alberch et al., 2002; Araujo and Hilt, 1997; Marco et al., 2002; McBride et al., 2003; Perez-Navarro et al., 2000; Perez-Navarro et al., 1999; Perez-Navarro et al., 1996; Ramaswamy et al., 2007). Later studies using either AAV vectors or transplanted mouse neural progenitor cells engineered to deliver GDNF or NRTN into the striatum of a transgenic HD mouse model (the N171-82Q mouse) found positive effects on striatal neuronal survival as well as on motor function in this model (Ebert et al., 2010; McBride et al., 2006; Ramaswamy et al., 2009). One study however using another transgenic HD mouse model (the R6/2 model) and delivery of GDNF by lentiviral vectors did not show any positive effects and the authors speculated that perhaps the intervention was initiated at a late disease stage, as this model is known to be rapidly progressive leading to premature death (Popovic et al., 2005). Taken together, the majority of published studies show that GDNF promotes survival of striatal neurons in rodents. Hence, effects on striatal neuronal function need to be considered in the delivery of these growth factors in the context of PD.

Another challenging aspect in scaling up from pre-clinical disease models is achieving sufficient vector transduction. Typically, high transduction efficiency is most satisfactorily achieved in rodent models. Scale-up to NHP results in partial coverage of the target areas, and in the few human post-mortem brains available, the transduction appears limited to even a smaller volume fraction of the target which might be sub-optimal. Experimentally, the volume occupied by vector can be increased by blocking target receptors of the vectors in the region by co-infusion of cell surface binding molecules specific to that serotype, e.g. heparin, laminin receptor, sialic acid, glycans (Akache et al., 2006; Kaludov et al., 2001; Shen et al., 2011; Summerford and Samulski, 1998; Wu et al., 2006). AAV2, and neurotrophic factors such as GDNF and NRTN, can bind to heparin-sulfate proteoglycans on the cell surface and co-infusion with heparin would therefore compete with this binding and increase the spread of vector (Hamilton et al., 2001; Nguyen et al., 2001). Their implementation in humans may however be complicated with other actions of these agents.

The pre-clinical evidence for efficacy of CERE-120 is extensive, with experiments in rodents, young NHPs, aged NHPs and MPTP-injected NHPs and the clinical results were still negative. Essentially all AAV gene therapy trials in PD patients (in addition to the CERE-120 discussed above, also the AAV-AADC and the AAV-GAD clinical trials) used the highest dose tested in NHP models as the highest dose tested in the Phase1 studies (Tables 1–2), and did not scale up to take into account the larger target volume in the human brain. Of note, the human putamen is more then 3-fold larger than the putamen of rhesus macaques, and around 5-fold larger than cynomolgus macaques (Yin et al., 2009). More importantly, the difficulty of scaling impacted on the Phase II studies as well, since the same dose was also used at this step. Notably, although the final Phase IIb trial testing efficacy of CERE-120 used a higher number of genomic copies/mm^3 targeted and still did not yield successful results. It would therefore be important to explore ways to incorporate a dose escalation study design (in Phase I trials) that reaches to the dose anticipated to replicate the coverage and concentration accomplished in animal models and show that the intervention is safe at this dose, thus enabling it to be tested for efficacy at the right dose level.

The volume occupied by vector in the target region is affected by the titer of the clinical grade vector used. Some of the processes used in laboratory grade production of vectors are either not scalable or not GMP compliant. Thus, the two production methods are at present not identical. These differences are known to have an impact on the final concentration of the vector preparation as well as its biological activity. It is therefore possible that experiments in small animal species using laboratory grade vectors and the vector that is made for use in humans do not have the same characteristics making the extrapolations from experimental studies and clinical trials even harder. In our opinion replication of key data in animals using the clinical grade vector should be implemented more widely and the results of this "pre-clinical efficacy validation of the clinical vector batch" should form part of the final decision process for initiation of clinical trials.

The injection rate can increase spread and reduce the number of injection sites and total surgery time but can also increase the risk for a host's immune response. Conversely, keeping volumes and rates constant while increasing injection sites can result in impractical surgery durations when translating from rodents to humans and also increase the risk of adverse effects related to multiple needle penetrations, hemorrhage, blood brain barrier disruption and inflammation. Further work is required to find optimal relationship between these parameters in going forward especially for administration in the brain, where the freedom to manipulate the variables relating to the vector injection are significantly restricted with the nature and sensitivity of the target organ.

The lack of efficacy in the CERE-120 trials were suggested to result from impaired axonal transport as nigrostriatal neurons lost their connections to their appropriate targets in the striatum and where the therapeutic factor was delivered. In a recent study, Kordower and colleagues showed that post-mortem tissue from PD patients at 4 years post-diagnosis and later have complete loss of staining for DA markers in the putamen (Kordower et al., 2013). Many of the recruited PD patients in the

Table 2
AAV-GDNF: Summary of study design for pre-clinical and clinical studies. All studies utilized AAV serotype 2 where GDNF was under a CMV promoter.

Study type	Disease stage	Subjects to treat	Side	Target nucleus	Total volume injected per hemisphere (μl)	Total genomic copies injected per hemisphere	Outcome	Reference	ClinicalTrials.gov identifier
Pre-clinical	*Macaca mulatta* (MPTP injected)	n = 6	Bilateral	Pu	150	5.0E11	*Increased FMT uptake in the AAV2-GDNF treatment group	Eberling et al. (2009)	
	Macaca mulatta (aged)	Low dose n = 3	Bilateral	Pu	75	8.3E10	*Increased FMT uptake in putamen and improved locomotor activity but no significant effect on ipsilateral dopamine levels	Johnston et al. (2009)	
		High dose n = 5	Bilateral	Pu	75	8.3E11			
		High dose n = 3	Bilateral	SN	50	5.5E11			
	Macaca mulatta (MPTP injected)	n = 8	Bilateral	Pu	150	9.9E11	* Improved motor function * Restoration of dopaminergic fibers in vector-treated putamen	Kells et al. (2010)	
Phase I	H&Y > 3 OFF medication, >30 UPDRS OFF medication	Dose 1 n = 6	Bilateral	Pu	450	9.0E10	*Ongoing		NCT01621581
		Dose 2 n = 6	Bilateral	Pu	450	3.0E11			
		Dose 3 n = 6	Bilateral	Pu	450	9.0E11			
		Dose 4 n = 6	Bilateral	Pu	450	3.0E12			

Abbreviations: AAV: adeno-associated viral vector, GP: globus pallidus, CMV: cytomegalovirus, WPRE: wood-chuck hepatitis post-regulatory element, MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Pu: putamen, Ca: caudate, SN: substantia nigra, STN: subthalamic nucleus, FMT: 6-[18F]-fluoro-L-m-tyrosine, CRS: clinical rating scale, UPDRS: Unified Parkinson's disease Rating Scale, H&Y: Hoehn and Yahr score.

trials described were at later stages of the disease, thus, there were probably very few DA terminals in the putamen to transport GDNF retrogradely and support remaining DA neurons. Recent experimental studies in NHPs injected with AAV-GDNF into the putamen with severe MPTP-lesions showed detectable GDNF in SN most likely via anterograde transport along the striato-nigral projection system (Kells et al., 2010). Future clinical trials will tell if the anterograde transport observed in animals can also occur in PD patients and whether this is a viable route to deliver biologically active GDNF close to the cell bodies of nigro-striatal projection neurons. Furthermore, at present we do not have any data in humans that would clarify if such an effect would be dependent on the stage of the disease and therefore the integrity of the remaining targeted neurons.

As all clinical trials in PD to date used recombinant AAV2 vectors, there has not been sufficient consideration regarding the choice of capsid serotype for this indication. The decision on the AAV capsid serotype going into clinical trials is driven not by an active selection of the best known serotype, rather due to circumstances around availability of GMP compliant production procedures and purification methods, contract manufacturing organizations with track-record in making these vectors, prior documentation of safety in humans, and likely also the intellectual property rights and protections surrounding the various serotypes that could be considered. This choice has a significant baring both on the extent of the transduction in the brain and also key indicators, such as the bio-distribution in the target region, capability of anterograde or retrograde transport of the viral particles themselves; site-specific versus widespread transduction and other aspects relating to reduced or completely abolished immune recognition of the particles in the recipient by modified capsids.

The pattern of release of the growth factor in the targeted area is another important aspect that should be considered. In the developing brain, it is well known that growth factors are expressed and released at well-determined time points when they are needed and the expression goes down once the intended target innervation patterns are accomplished (Gates et al., 2004; Kumar et al., 2015; Lopez-Martin et al., 1999). It is possible that regulation of gene expression would be required both to express these potent molecules at physiologically relevant ranges and to mimic the natural pattern of release in order to obtain the intended neuroprotective and neurorestorative effects in the aged diseased brain. In fact, when high and low levels of expression of GDNF from AAV vectors were compared in the rodent and NHP brain, it became clear that it is possible to overdose the system and trigger compensatory changes at least in the rodent brain and that the low-dose expression provided excellent results in the NHPs (Eslamboli et al., 2005b; Georgievska et al., 2004b; Rosenblad et al., 2003). Several regulatory elements have already been tested in animals to control the level of GDNF expression using transcriptional and translational regulatory elements or more recently control of protein stability in the cells (Barroso-Chinea et al., 2016; Chtarto et al., 2007; Georgievska et al., 2004a; Quintino et al., 2013; Tereshchenko et al., 2014). These approaches are likely to be incorporated in future clinical trials and give better control over the amount and duration of expression in humans. They will also allow the clinicians to test if the expression needs to be maintained continuously over a long period, or could be equally or more effective when provided as repeated pulsatile administrations.

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