

Viral vector delivery of neurotrophic factors for Parkinson's disease therapy

MARTIN J. KELLY, GERARD W. O'KEEFFE, AIDEEN M. SULLIVAN

Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

Parkinson's disease (PD) is a neurodegenerative disorder characterised by the progressive loss of midbrain dopaminergic neurons, which causes motor impairments. Current treatments involve dopamine replacement to address the disease symptoms rather than its cause. Factors that promote the survival of dopaminergic neurons have been proposed as novel therapies for PD. Several dopaminergic neurotrophic factors (NTFs) have been examined for their ability to protect and/or restore degenerating dopaminergic neurons, both in animal models and in clinical trials. These include glial cell line-derived neurotrophic factor, neurturin, cerebral dopamine neurotrophic factor and growth/differentiation factor 5. Delivery of these NTFs via injection or infusion to the brain raises several practical problems. A new delivery approach for NTFs involves the use of recombinant viral vectors to enable long-term expression of these factors in brain cells. Vectors used include those based on adenoviruses, adeno-associated viruses and lentiviruses. Here we review progress to date on the potential of each of these four NTFs as novel therapeutic strategies for PD, as well as the challenges that have arisen, from pre-clinical analysis to clinical trials. We conclude by discussing recently-developed approaches to optimise the delivery of NTF-carrying viral vectors to the brain.

Dopaminergic neurotrophic factors (NTFs)

NTFs are proteins which promote the survival and healthy development of neurons; the effects of these factors are mediated via stimulation of multiple intracellular signalling pathways in these cells (for reviews see Refs 1, 2, 3). NTFs which act on dopaminergic neurons have been extensively studied as therapeutics for Parkinson's disease (PD), since this neurodegenerative disorder is caused by the death of dopaminergic neurons projecting from the substantia nigra in the midbrain to the caudate-putamen in the forebrain. Loss of these neurons results in the characteristic motor symptoms of PD-bradykinesia, rigidity and resting tremor. NTFs have potential to protect and restore degenerating dopaminergic neurons, thus slowing or halting the disease progression. NTFs which have potent dopaminergic neuroprotective effects include glial cell-line derived neurotrophic factor (GDNF), neurturin (NRTN) and growth/differentiation factor 5 (GDF5). These NTFs belong to the transforming growth factor- β (TGF β) protein superfamily. The neurotrophic effects of GDNF, NRTN and GDF5 on the nigrostriatal pathway have been studied for more than a decade; both GDNF and NRTN have been tested in clinical trials (for review see Ref. 4). However, these proteins are not the only NTFs being explored for their potential in PD. Cerebral dopamine neurotrophic factor (CDNF) and its protein paralogue, mesencephalic astrocyte-derived neurotrophic factor (MANF), have recently been

demonstrated to confer neurotrophic and restorative actions on the nigrostriatal pathway (for review see Ref. 5).

NTFs exert their effects by binding to their individual receptors on the neuronal cell membrane, then triggering specific intracellular signalling cascades. For many TGF β superfamily members, signalling is initiated through the ligand binding to a type-2 serine/threonine receptor. The resulting ligand-receptor complex recruits and binds to the corresponding type-1 serine/threonine receptor. Phosphorylation of the type-1 receptor at a glycine-serine-rich domain by the type-2 receptor promotes downstream activation of receptor-Smads (R-Smads). The R-Smads bind to a co-Smad, Smad-4, which facilitates entry of the complex into the nucleus and thus regulates the transcription of a variety of genes (for reviews see Refs 1, 6). In the case of GDF5, the type-1 and type-2 receptors most commonly used are bone morphogenetic protein receptor (BMPR)-1b and BMPR-2, respectively (Ref. 7). The resulting activated R-Smads are Smad-1, Smad-5 and Smad-8.

GDNF and NRTN signalling occurs via a different mechanism to that of GDF5 and other TGF β s. These two factors act by activating a receptor tyrosine kinase, Ret, after forming a ligand-receptor complex with a glycosylphosphatidylinositol-anchored GDNF family receptor α (GFR α). GDNF preferentially binds to GFR α 1, while NRTN preferentially binds to GFR α 2. Once Ret has been stimulated by the

ligand–receptor complex, a Ret–Ret homodimer is formed and is auto-phosphorylated. Downstream intracellular signalling pathways activated by phosphorylated Ret include those involving mitogen-activated protein kinase, c-Jun N-terminal kinase (JNK), Src kinases, Akt-phosphoinositide-3 kinase and phospholipase C γ (for reviews see Refs 2, 3).

Potential of dopaminergic NTFs for treatment of PD

There is a pressing need for disease-modifying therapies for PD, since currently-used treatments focus on symptom management rather than on their cause. NTFs hold significant promise in this respect. The most widely-used pharmacological approach is administration of levodopa (L-DOPA), a precursor of dopamine, to replenish diminished levels of striatal dopamine. However, at least half of L-DOPA users experience drug-induced dyskinesia or other motor complications after about 5 years of therapy. Agents which are used in combination with L-DOPA to optimise its effects include DA agonists, peripheral aromatic amino acid decarboxylase inhibitors, monoamine oxidase B inhibitors and catechol-O-methyl transferase inhibitors (for reviews see Refs 8, 9). Alternatives to pharmacological treatment, for patients in which L-DOPA therapy does not work well or no longer works, include deep brain stimulation (DBS). DBS confers improvements in motor symptoms due to ablation of brain structures such as the thalamus or pallidum. This strategy is not without complications and is typically used as a last resort to manage motor symptoms (Ref. 10). Like L-DOPA, DBS does not halt or slow the progression of the neurodegeneration. Thus, other approaches, including cell-based therapies and NTFs, are under intensive investigation as disease-modifying therapies.

The use of NTFs has the potential advantage that these factors may protect the remaining nigrostriatal dopaminergic neurons from delayed or further damage, rather than only treating the disease symptoms. This is crucial, as approximately 50% of these neurons have degenerated, corresponding to a loss of striatal input of about 80%, by the time that PD symptoms are clinically apparent (for review see Ref. 11). Since approximately 90% of PD cases are of idiopathic origin, earlier detection of PD in the general population is a difficult challenge to overcome at present. Thus the use of NTFs as an intervention in the neurodegenerative process upon PD diagnosis has the potential to be a very beneficial approach.

Application of dopaminergic NTFs as recombinant proteins

GDNF

GDNF is the most extensively studied dopaminergic NTF to date. In vitro studies highlighted GDNF's neurotrophic effects on developing dopaminergic

neurons (Refs 12, 13, 14). GDNF treatment enhanced neuritic branching in cultured embryonic rat dopaminergic neurons (Ref. 14) and protected them against death induced by the neurotoxins 1-methyl-4-phenylpyridinium (MPP⁺) (Ref. 15) and 6-hydroxydopamine (6-OHDA) (Ref. 16), in vitro models of the neurodegeneration that occurs in PD. In vivo, GDNF has been shown to bestow significant neuroprotection on adult rodent midbrain dopaminergic neurons against 6-OHDA lesions in short-term (Refs 17, 18, 19, 20, 21) and long-term (Ref. 22) studies (for reviews see Refs 4, 23). Such neuroprotection was also observed when GDNF was administered prior to or following injection of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouse midbrain (Ref. 24). Delayed injection of GDNF after 6-OHDA lesions can confer significant restorative effects on the host striatum (Refs 25, 26). Furthermore, GDNF treatment enhances the survival and function of embryonic rat dopaminergic grafts in 6-OHDA-lesioned rats (Refs 27, 28, 29, 30). Lastly, GDNF treatment can induce neuroprotective effects in monkey parkinsonian models (Refs 31, 32, 33, 34, 35, 36).

Due to the encouraging performance of GDNF in both in vitro and animal studies, intracerebral application of this protein was examined in human clinical trials. Since NTFs do not cross the blood–brain barrier and are rapidly biometabolised in vivo, clinical application requires them to be injected intracerebrally. In the initial clinical trial, recombinant human GDNF (Liatemin[®]; Amgen Incorporated) was infused via an intraventricular route in 38–50 patients undertaking a randomised, placebo-controlled double-blind trial (Ref. 37). No significant increases in the Unified Parkinson's Disease Rating Scale (UPDRS) scores were observed and participants experienced undesirable symptoms ranging from appetite loss to depression and paraesthesia (Ref. 37). The poor outcome of this trial may have been due to the fact that delivery was intraventricular. More positive results were achieved following GDNF administration directly to the brain parenchyma, in two open-label trials in the UK (Refs 38, 39) and the USA (Ref. 40). Both of these studies infused GDNF into the putamen, and observed significant overall improvements in the UPDRS scores for both on- and off-medication phases in patients with advanced PD. These patients did not suffer any significant side-effects, apart from a mild Lhermitte's sign (an electrical sensation that runs down the back and into the limbs). The success of these open-label trials led to the instigation of a randomised, placebo-controlled trial using intraputaminally delivered GDNF (Ref. 41). Unfortunately the promising therapeutic effects seen in the open-label trials were not reproduced. Furthermore, safety issues were raised, as some patients developed antibodies towards the exogenous human GDNF (Ref. 42). In 2013, following a safety trial in six PD patients, the Bristol group initiated a phase 2 clinical trial involving 36 patients, implementing a

new infusion strategy for GDNF protein. This ongoing trial uses an optimised delivery port and the researchers are hopeful that this will circumvent some of the problems encountered in the previous trials.

NRTN

NRTN is a member of the GDNF family of proteins (Ref. 43), and has been shown to exert neurotrophic effects on embryonic rat dopaminergic neurons in vitro, to the same extent as GDNF (Refs 44, 45). Unlike those treated with GDNF, however, NRTN-supplemented neurons did not display extensive branching (Ref. 45). The impacts of NRTN on survival and branching of nigral dopaminergic neurons in a 6-OHDA rat model of PD reflected those observed in the in vitro study (Ref. 45). Another in vivo study showed that NRTN protected the function of nigrostriatal neurons, as expressed in terms of amphetamine-induced rotational asymmetry, following 6-OHDA-lesioning of the adult rat medial forebrain bundle (MFB) (Ref. 44) or striatum (Refs 46, 47). Rosenblad et al. (Ref. 46) observed that injection of NRTN into the striatum or ventricles conferred a lower level of dopaminergic neuroprotection than that achieved after intranigral injection, as reported by Horger et al. (Ref. 44). The level of protection conferred was also lower than that achieved by intrastriatal GDNF, suggesting that NRTN has poorer solubility and diffusion properties in vivo (Ref. 46).

The neuroprotective effects of intraputaminial NRTN infusion have also been tested in primates. Chronic infusion of NRTN in MPTP-lesioned rhesus monkeys resulted in an improved parkinsonian motor score (Ref. 48), albeit a lower effect than that conferred by GDNF infusion in a previous study (Ref. 33). The number of dopaminergic neurons in the substantia nigra of NRTN-treated monkeys did not differ significantly from that of MPTP-only group (Ref. 48), again highlighting the issue of poor diffusion of NRTN (Ref. 46). Clinical trials of NRTN protein have not been conducted, probably because of the safety issues that arose during the GDNF trials (Refs 41, 42). However, trials using NRTN with adeno-associated virus (AAV) type-2 (AAV2) have been completed, as discussed below.

CDNF

Preliminary findings from an in vitro study of MANF (Ref. 49) led to the development of in vivo studies of its paralogue, CDFN, in animal models of PD. The initial study described MANF as an astrocyte-derived factor, which had selective protective actions on dopaminergic neurons in culture (Ref. 49). Thus, CDFN was proposed as a novel NTF for PD treatment. Subsequent analysis led to the categorisation of a new family of proteins consisting of CDFN and MANF (Refs 50, 51). The neurotrophic potential of CDFN was then demonstrated in an in vivo rat model of PD, the intrastriatal 6-OHDA lesion (Ref. 50). Unilateral intrastriatal

CDFN given 6 h prior to 6-OHDA conferred neuroprotective effects at least as potent as those of GDNF (Ref. 50). CDFN administration 4 weeks after striatal 6-OHDA lesioning resulted in partial neurorestoration of the nigrostriatal system (Ref. 50). In mice, intrastriatal injection of CDFN just prior to, or 1 week after, peripheral MPTP injection, conferred dopaminergic neuroprotective or restorative properties, respectively (Ref. 52). Chronic intrastriatal infusion of recombinant human CDFN over 2 weeks in 6-OHDA-lesioned rats induced cumulative improvements in motor behaviour and preservation of nigral dopaminergic neurons, to a greater extent than GDNF infusion (Ref. 53). This study also demonstrated that radiolabelled CDFN protein followed a similar retrograde transport profile to that of GDNF, being transported to the nigra when administered via intrastriatal infusion (Ref. 53). No chronic infusion approach of CDFN protein has so far been explored in primate models of PD or in clinical trials. Nevertheless, viral vector-mediated CDFN research is underway, with in vivo studies examining the effects of AAV2-mediated CDFN delivery to the nigrostriatal pathway in animal PD models, as discussed below (Refs 54, 55).

GDF5

Like GDNF, the neurotrophic and neuroprotective effects of GDF5 on dopaminergic neurons have been demonstrated in vitro and in vivo. The first study showed that treatment with recombinant human GDF5 protected cultured rat dopaminergic neurons from MPP⁺-induced damage (Ref. 56). GDF5 treatment was further found to promote dopaminergic neuronal survival and stimulate their neurite outgrowth in vitro (Refs 57, 58, 59, 60), properties which are of value to its therapeutic application. Pretreatment with recombinant human GDF5 had survival-promoting and functional effects on embryonic rat dopaminergic neurons after transplantation to the 6-OHDA-lesioned rat striatum; these neuroprotective effects were comparable with those seen after GDNF pretreatment (Ref. 29). In adult rats, intracerebral injection of GDF5 protected nigrostriatal dopaminergic neurons against 6-OHDA lesions (Refs 61, 62). The efficacy of GDF5 was highest after injection into the striatum and/or nigra, in comparison with intraventricular delivery (Ref. 62). Thus, although intraventricular administration of NTFs would be less invasive in a clinical setting, it appears that direct administration to the parenchyma is the most efficacious approach. For NTFs to be used clinically in PD patients, they would be administered after significant striatal denervation had already taken place. Thus, it is important to test the effects of potentially therapeutic factors when they are administered after a nigrostriatal lesion, in animal models. Promising results were reported in a study which achieved restoration of nigrostriatal function when recombinant GDF5 was administered at 1 or 2 weeks after intrastriatal 6-OHDA (Ref. 63). A greater

effect was seen when GDF5 was administered after a 1-week delay than after 2 weeks (Ref. 63), highlighting the issue that timing is important in the clinical setting.

Potential for gene therapy in NTF therapy for PD

The more advanced the disease by the time of NTF administration, the less effective the therapy is likely to be. At least until earlier diagnosis of PD is possible, it is critical that NTF therapy is optimised to ensure the best possible clinical efficacy, even in the advanced disease state. Since administration of NTF proteins is hampered by their rapid metabolism in the brain, methods which ensure a consistent supply of NTF to dopaminergic neurons are desirable. Overexpression of NTFs using a gene therapy approach may be the optimal manner in which to ensure an adequate supply to the degenerating nigrostriatal neurons (Fig. 1). Gene therapy has previously been applied in patients with advanced-stage PD, to deliver the enzyme glutamic acid decarboxylase; this was the first double-blind clinical trial to show an effect of gene therapy in a neurological disease (Ref. 64). Thus, the gene therapy approach has been proven to be safe and effective in PD.

Viral vectors used in gene therapy

Viruses are naturally efficient at introducing genes into target cells. There are several viral vectors which possess natural tropism for fully mature neurons; each type has individual benefits and shortfalls (Fig. 2). Viruses that have been used in development of vectors for NTF delivery include adenovirus (AdV), AAV and lentivirus (LV) (for reviews see Refs 23, 65, 66). Each of these is capable of delivering their genome into nondividing cells such as neurons, supporting their use in neuronal gene therapy. However, size limits exist on the viral capacity to carry transgenes. AdV and LV viruses possess much larger capacities than AAV for transporting recombinant DNA. To provide an effective, efficient, long-term treatment approach, stable integration into the host DNA is sought. Unlike AAV and LV, AdV does not integrate into the host genome. AdV has another disadvantage in that it can induce dose-dependent inflammatory responses in hosts. With regards to vectors that can be integrated, LV vectors ensure much earlier protein expression than AAV vectors (hours versus several days; for review see Ref. 65). For AAV-delivered NTFs, this additional time needed for transgene expression must be considered when assessing the usefulness of the system to deliver NTFs in a clinical paradigm. Some LV vectors present a potential tumour-producing risk, due to the manner in which they integrate into the genome. AAV vectors, in contrast, are deemed to be safe for clinical use. Finally, AAV has a diffusion advantage over AdV and LV due to its particle size, which is 25 nm in diameter, compared with at least 70 nm for AdV particles, whereas LV particles are no

less than 100 nm in diameter. While AdV has been the most frequently chosen virus to date for all clinical trials (for review see Ref. 67), AAV2 has been the virus of choice for developing gene therapeutics for PD.

Gene therapy approaches to deliver GDNF, NRTN, CDNF and GDF5

GDNF

GDNF-expressing vectors have been designed and tested in animal studies using AdV, LV and AAV (for review see Ref. 23). GDNF delivered by AdV vector to the substantia nigra (Refs 68, 69) or striatum (Refs 70, 71, 72) of rats with intrastriatal 6-OHDA lesions induced motor improvements and protection of nigral dopaminergic neurones. When administered into the substantia nigra after a 6-OHDA MFB lesion, AdV-delivered GDNF also resulted in significant motor improvements (Ref. 73), whereas in the intrastriatal lesion model, it was effective when injected into the substantia nigra, but not the striatum (Ref. 74). In MPTP-treated mice, AdV-mediated GDNF delivery to the striatum prevented depletion of striatal dopamine levels (Ref. 75).

Administration of AAV–GDNF into the rat striatum, but not substantia nigra, 4 weeks prior to 6-OHDA lesion surgery resulted in significant improvements on amphetamine-induced rotational tests (Ref. 76). In marmosets, injection of AAV2–GDNF 3 weeks before 6-OHDA-lesion surgery induced significant reductions in amphetamine-induced rotations (Ref. 77). In these AAV2–GDNF-treated monkeys, dopaminergic neuronal sprouting was observed in the lesioned striatum, as well as significant sparing of dopaminergic neuronal cell bodies in the substantia nigra (Ref. 77). Both of these studies involved the injection of the therapeutic vector prior to administration of the dopaminergic toxin. However, in order for viral vectors to be therapeutically applicable, they need to be shown to induce restoration of nigrostriatal function when administered after the lesion.

LV-based vectors have also been used to deliver GDNF in animal models of PD, conferring neuroprotective effects on the nigrostriatal pathway of 6-OHDA-lesioned rodents (Refs 78, 79) and MPTP-treated primates (Ref. 80). Intrastriatal delivery of LV-GDNF was effective in reducing 6-OHDA-induced damage to nigrostriatal neurons, protecting nigral dopaminergic neuronal cell bodies and resulting in sprouting of their lesioned axons along the nigrostriatal pathway (Ref. 79). This study reported that LV-driven GDNF expression was maintained for at least 8 months *in vivo*, and that GDNF protein was anterogradely transported to the nigra from the intrastriatal injection site. Another study showed that LV-GDNF protects 6-OHDA-lesioned rats from deficits in an operant task which measures complex motor functions, as well as deficits in several tests of simple motor function (Ref. 81). LV-GDNF was also shown

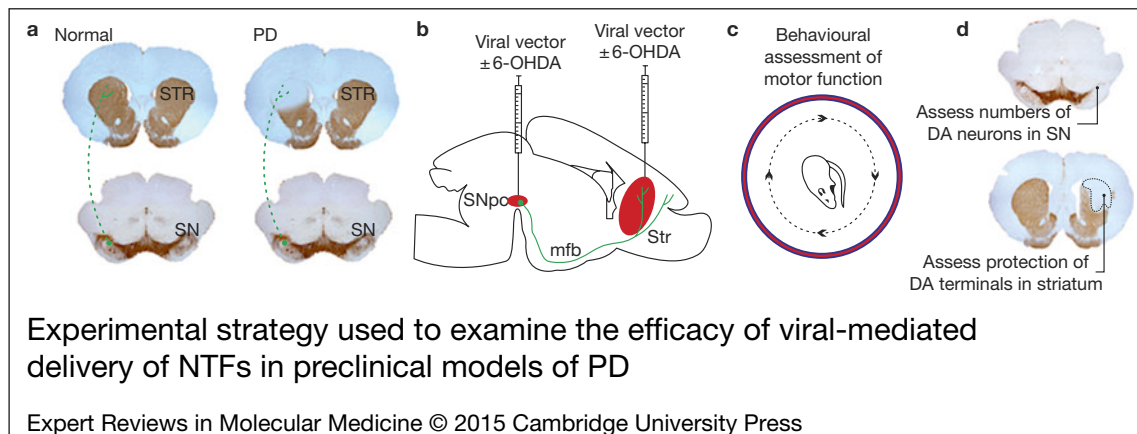


FIGURE 1

Experimental strategy used to examine the efficacy of viral-mediated delivery of NTFs in preclinical models of PD. (a) PD results from a loss of dopaminergic neurons in the SN which leads to a reduction in striatal dopaminergic innervation. (b) Preclinical assessment of the neuroprotective efficacy of viral-mediated delivery systems involves stereotactic administration of the viral vector to the striatum or midbrain, prior to, at the same time, or after, stereotactic administration of a neurotoxin (e.g. 6-OHDA) to either the (SNpc), striatum (Str) or medial forebrain bundle (mfb). (c) Assessment of motor function after surgery, coupled with (d) post-mortem assessment of numbers of dopaminergic neurons and their striatal processes, is used to determine efficacy of viral-delivered NTFs.

to induce reinnervation of the partially denervated striatum, and to restore motor function, when injected 4 weeks after intranigral 6-OHDA (Ref. 82).

In contrast to the promising results of studies that applied GDNF in 6-OHDA-lesioned rats, a recent study found that GDNF delivery by either AAV or LV vectors was not effective in preventing or minimising neurodegeneration caused by an AAV- α -synuclein (AAV- α -syn) vector (Ref. 83). Mutations in α -synuclein are associated with autosomal dominant PD (for reviews see Refs 84, 85). Overexpression of α -synuclein using AAV is a recently developed animal model of PD, in which protracted and progressive degeneration of the nigrostriatal pathway is observed (for review see Ref. 86). This model has an advantage over the 6-OHDA model as it mimics some of the pathology of the human disease. An LV-GDNF vector and an AAV-GDNF vector were separately tested in AAV- α -syn-treated adult rats (Ref. 83). LV-GDNF was administered 2 weeks prior to the AAV- α -syn injection, whereas AAV-GDNF was administered 3 weeks beforehand (Ref. 83). Target sites for the therapeutic vector injections included the rostral substantia nigra, within the striatum, or both (Ref. 83). Neither LV-GDNF nor AAV-GDNF significantly reduced the dopaminergic degeneration induced by the AAV- α -syn vector, despite the observed overexpression of GDNF from both vector systems (Ref. 83). A subsequent study showed that α -synuclein overexpression downregulates the expression of Nurr1, and its downstream target, the GDNF receptor Ret (Ref. 87). Further investigation of the cellular mechanisms underlying the neuropathology observed in the AAV- α -syn rat model is warranted, in order to ascertain the validity of this model for future studies on NTFs. AAV-mediated overexpression of α -synuclein may result in cytosolic levels of α -synuclein that exceed those seen

in the human disease. Thus, it may be necessary to refine these models to more closely mimic the neuropathology of human PD. Clearly, the choice of animal model has a critical impact on whether a potential therapeutic may be considered for future clinical trials. The difference between the effects of GDNF treatment in the α -synuclein model and the more widely-used 6-OHDA and MPTP models mandates considerable thought in the validation of therapeutics for PD. Indeed, it is possible that GDNF may not be the best choice of NTF for PD therapy, since the expression of its receptor is downregulated by α -synuclein. Strategies to circumvent this limitation could involve attempts to upregulate Nurr1, in combination with optimised GDNF delivery to the nigrostriatal system, as discussed below. However, it may be that GDNF is not the best factor for clinical use, which strengthens the argument for investigation of other dopaminergic neurotrophic factors that act via receptors that are not affected by the disease pathology.

In terms of optimised delivery, several recent studies by the Bankiewicz group, using a system of convection-enhanced delivery (CED) to deliver AAV2-GDNF in animal models, have shown promise (Refs 88, 89, 90, 91, 92, 93). The use of CED as an alternative approach of viral vector delivery is relatively novel (for review see Ref. 94), although this approach had initially been used to demonstrate the delivery of macromolecules nearly two decades ago (Ref. 95). Johnston et al. (Ref. 89) began preliminary testing of AAV2-GDNF via CED in aged rhesus macaques, while a parallel study used MPTP-lesioned rhesus macaques (Ref. 88). In the first study, the safety of this approach was confirmed, as was very effective delivery of GDNF protein to the target regions (Refs 89, 92). Low-dose, unilateral putaminal infusion of AAV2-GDNF slowed the decline in locomotor

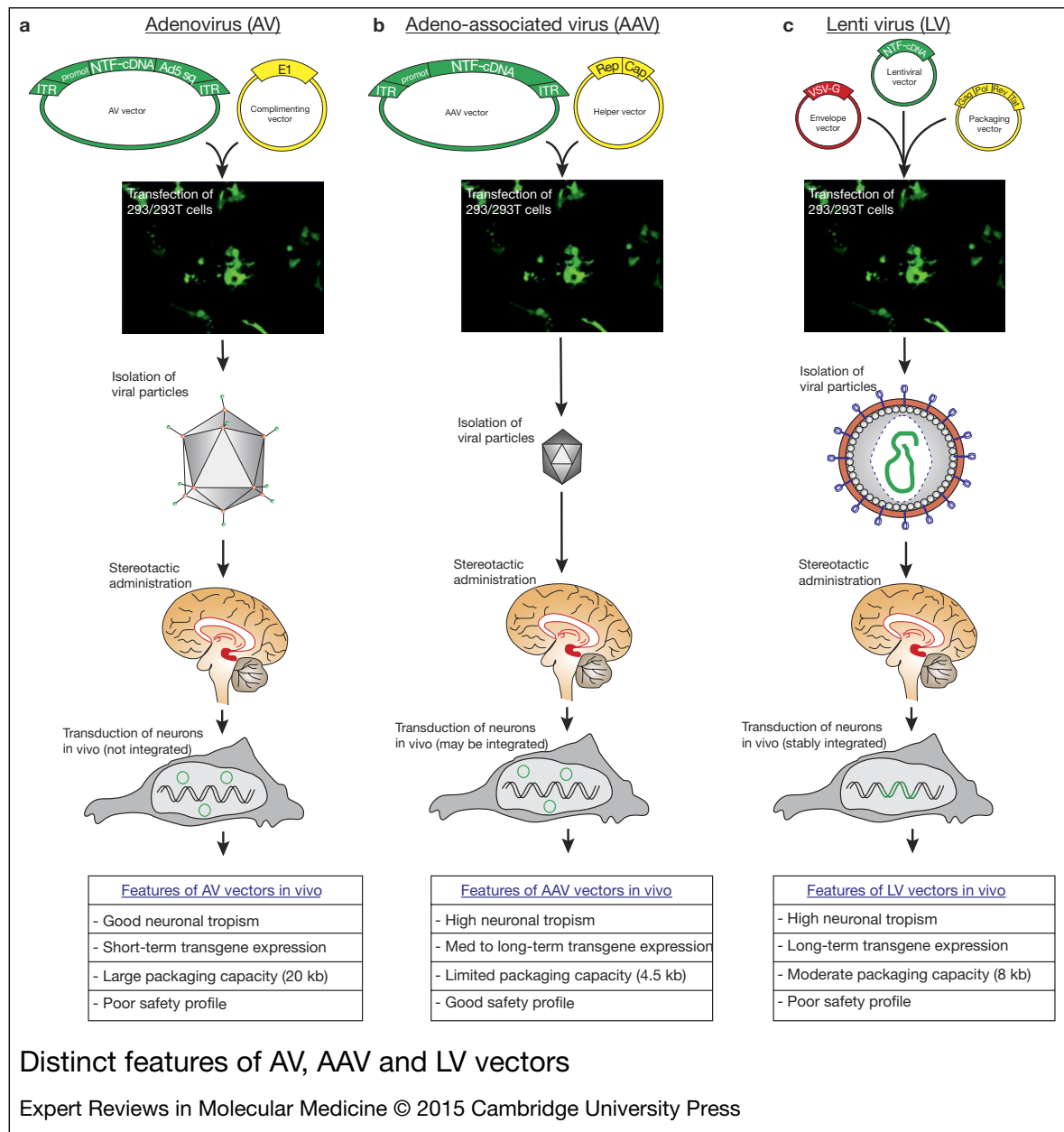


FIGURE 2

Distinct features of AV, AAV and LV vectors. AdV, AAV and LV vectors have all been explored for the delivery of NTFs in PD models. Panels (a–c) show the events involved from in vitro generation to stereotactic administration of each viral vector type. Shown also is an overview of the distinct features of each viral vector when applied to the brain in vivo.

activity that is a normal feature of aging in these monkeys (Ref. 89). Efficient transport of GDNF to the caudate, as well as putamen, was achieved following intranigral infusion of the vector, indicating anterograde transport of GDNF along the nigrostriatal pathway. This study also found that AAV2-GDNF induced upregulation of tyrosine hydroxylase (TH) expression, significant increases in the size (but not number) of nigral dopaminergic cell bodies, and enhancement of dopaminergic function in the ipsilateral putamen (Ref. 89). Although this study was not performed in a PD model, the confirmation of safety and effective delivery in the aged monkey brain was an important step towards the clinical application of

this delivery approach in PD patients, the majority of whom would be of advanced age. Encouraging results were also seen in the parallel study, which used MPTP lesions to induce a model of early stage PD in one hemisphere and advanced-stage PD in the other hemisphere, in rhesus monkeys (Ref. 88). Infusion of AAV2-GDNF via CED 4 months after establishment of the lesions induced significant improvements in clinical rating scale scores over the following nine months, compared with control animals that had received saline by CED (Ref. 88). A significant increase in dopaminergic function, as measured by PET, was observed in both mildly- and severely-lesioned hemispheres at 6 months

post-treatment, compared with that in controls (Ref. 88). As with the companion study performed in aged monkeys, the safety of this GDNF delivery approach was confirmed (Ref. 92). This study, by showing the efficacy of AAV2-GDNF in already-lesioned animals, provides important support for the eventual clinical application of CED-mediated AAV2-GDNF in PD patients. The long-term neurorestorative capability of CED-mediated AAV2-GDNF in this group of MPTP-lesioned primates was verified after 24 months (Ref. 90). The improvements in motor function and striatal dopaminergic function which had been recorded after 6 and 12 months in the earlier study (Ref. 88) were maintained for 24 months (Ref. 90). Post-mortem analysis showed increases in TH-immunopositive fibre density and sprouting in the lesioned putamen (Ref. 90). Importantly, no significant adverse effects were recorded in this study, indicating good tolerance of high-dose GDNF delivery to the putamen in the long term. The only concern was weight loss, and dyskinesia in a leg of one monkey, which the authors attribute to mispositioning of a cannula in this animal (Ref. 90). Weight loss issues following GDNF treatment have been noted in other studies (for examples, see Refs 34, 36, 37, 92, 96, 97). This highlights the importance of optimal positioning of delivery cannulae for safe and accurate delivery of NTFs in future clinical studies. Another important point to be raised by the above studies is that anterograde transport of GDNF or AAV-2-GDNF particles appears to be the predominant manner in which GDNF is conveyed in the brain following intracerebral administration (Refs 89, 90). This suggests that intraputaminial infusion of AAV2 vectors for delivery of NTFs is the most appropriate, as it will allow transport of the vector and/or factor to the nigra along striatonigral axonal projections, which are largely intact in PD patients, even when nigrostriatal projections have severely degenerated. It is worth noting that these studies by the Bankiewicz group investigated the regenerative effects of GDNF in the already-lesioned monkey brain, in contrast to the majority of studies, which examined the protective effects of NTFs delivered prior to the lesion. Although there have been some studies reporting the restorative effects of GDNF (Refs 25, 26), CNTF (Refs 50, 52) and GDF5 (Ref. 63) in rodent PD models, there is a need to extend these studies to primate models. Optimisation and standardisation of study design in preclinical models will allow the results to be more applicable to clinical studies, which by necessity evaluate the restorative rather than protective effects of NTFs in the diseased brain.

With the issue of optimal positioning of delivery cannulae in mind, studies by the Bankiewicz group explored magnetic resonance imaging-guided CED of AAV2-GDNF in a preclinical setting, using gadoteridol (Gd) to assist in real-time tracking of the infusions (Refs 91, 93, 98). Gd was shown to be an accurate

tracer for the distribution of GDNF protein after CED-administered AAV2-delivery (Refs 91, 93, 98). Richardson et al. successfully tested the delivery of Gd using the ClearPoint System™ technology from SurgiVision Inc. (Ref. 99) and further validated this approach for AAV2-GDNF delivery (Ref. 91). Bankiewicz and co-workers have also carefully considered the surgical cannula design (Refs 100, 101, 102) and insertion zones (Refs 102, 103), to optimise the delivery of AAV vectors to the brain. Thus, this optimised method of CED-mediated delivery provides a useful tool to track the infusion of viral vectors to the brain, and has the potential to greatly enhance current delivery strategies for NTFs in PD. Since the main problems with the unsuccessful clinical trials to date appear to be related to insufficient or inappropriate delivery of the therapeutic factor to the target tissue, such advances in delivery methodologies should promote the drive towards the instigation of future trials.

Regulated delivery of the therapeutic gene of interest is of great value in PD, as it allows the opportunity to fine-tune the dose of NTF to be delivered, depending on the patients' responses. This can be achieved by peripheral administration of a drug which can enter the brain and regulate transcription of the therapeutic gene encoded by the viral vector. Hadaczek et al. used this approach to control GDNF delivery from a modified AAV-2 vector, in which GDNF transcription was regulated by the administration of rapamycin (Ref. 104). The therapeutic implications of this system have yet to be evaluated, but this approach holds great promise for the future widespread clinical use of viral vector-mediated NTF delivery.

NRTN

An initial report showed that intracerebral injection of LV-NRTN conferred neuroprotective effects on the rat nigrostriatal system against intrastriatal 6-OHDA (Ref. 105). Subsequently, AAV2-delivered NRTN, under the name CERE-120 (Ceregene Incorporated), was tested in 6-OHDA-lesioned rats (Refs 106, 107, 108) and MPTP-lesioned monkeys (Refs 109, 110). Both young (Refs 109, 111) and aged nonparkinsonian monkeys (Refs 109, 112) were used to test the safety and *in vivo* expression profile of CERE-120. Intrastriatal CERE-120 2 weeks prior to 6-OHDA conferred neuroprotection of rat nigral dopaminergic neurons, as well as stable expression of NRTN protein, for at least 1 year, with no adverse effects (Ref. 107). Monkeys which had received CERE-120 injections in both striatum and nigra 4 days after MPTP administration displayed significant improvements in parkinsonian clinical scores, as well as significant preservation of nigrostriatal dopaminergic neurons, during a 10-month study (Ref. 110). Both anterograde and retrograde transport of NRTN was detected by post-mortem analysis (Ref. 110). Significant increases in dopaminergic fibre density

were found in the ipsilateral striatum of aged monkeys at eight months after intrastriatal CERE-120 (Ref. 112). A further study showed the safety of intrastriatal CERE-120 in primates, following extensive toxicological and pathological analysis (Ref. 111). One result of concern which emerged was that CERE-120 resulted in downregulation of TH expression in rat nigrostriatal dopaminergic neurons, albeit without affecting their structural integrity. However, this effect was not observed in monkeys and was deemed to be a rodent-specific nontoxic compensatory response to the increase in dopaminergic tone induced by NRTN (for review see Ref. 113), as had been previously reported for GDNF (Ref. 114). Despite the variable statistical significance of these pre-clinical data, the indication that CERE-120 was safe, well-tolerated even at very high doses (for review see Ref. 113) and potentially efficacious in rodents and nonhuman primates ensured that it entered into clinical trials.

CERE-120 was tested in an open-label phase I clinical trial (Ref. 115) and then in a double-blind, randomised, controlled phase II trial (Ref. 116). The initial trial involved bilateral intraputamenal injection of one of two doses of CERE-120 to 12 patients suffering from advanced PD, with clinical follow-up for 1 year (Ref. 115). No serious adverse effects were reported; transient anti-AAV2 antibodies were produced but caused no symptoms (Ref. 115). The patients' motor symptoms and quality-of-life indices showed variable improvement levels, with the main benefit being reductions in their off-medication UPDRS scores, in the time spent in 'off' and in dyskinesias (Ref. 115). Intraputamenal CERE-120 was thus deemed to be a safe and potentially effective treatment for PD, and a phase II study was initiated by Ceregene. The phase II trial involved 58 patients; one-third of these was designated to the control group and received sham surgery (Ref. 116). As in the phase I trial, some patients produced transient AAV2 antibodies, but none produced antibodies to NRTN (Ref. 116). Thirteen (out of 38) of the CERE-120-treated patients reported serious side-effects, but these were attributed to the surgical procedure rather than to the treatment, since they were also experienced by four (out of 20) of the control group (Ref. 116). The study also reported tumour formation in three of the CERE-120-treated patients, and in two control patients, which was not attributed to the surgical procedure (Ref. 116). The CERE-120-treated patients did not experience significant improvements in their UPDRS scores at 12 months after their initial assessment, compared with those of the control group. Thus, the phase II trial did not meet its primary endpoint and was at that point deemed to be a failure (Ref. 116). However, additional data presented from this study showed that the CERE-120-treated patients did show significant improvements in off-medication UPDRS score when assessed after 18 months, suggesting a delayed neurotrophic effect (Ref. 116).

Post-mortem analysis on two patients who had received CERE-120 (and died of unrelated causes at 1.5 and 3 months after the trial) highlighted important differences in the distribution of AAV-2 mediated NRTN delivery to the human and animal brains (Refs 109, 116). Bartus et al. examined the bioactivity and distribution of CERE-120 after intraputamenal delivery in young, aged and MPTP-treated rhesus monkeys, and compared these with the patterns found in the two post-mortem brains from the clinical trial (Ref. 109). They reported that in the PD patients, intraputamenal CERE-120 resulted in NRTN expression in ~15% of the putamen, but no expression in the substantia nigra. This is in contrast to their studies on the monkey brain, in which nigral expression of NRTN was seen after delivery solely to the putamen (Ref. 109). Any potential retrograde transport of NTN is hampered by its low level of diffusion and secretion, in contrast to GDNF, which is readily secreted and diffuses easily in brain parenchyma. It is likely that the restricted extent of putamenal transduction that was achieved in the Phase 2 trial, in combination with the limited diffusion of NTN protein, prevented any significant retrograde transport to the nigra. The lack of retrograde transport may be the reason for the absence of clinical benefit after intraputamenal delivery of CERE-120 in advanced-stage patients (Ref. 116). This highlights the importance of early diagnosis of PD to enable treatment while the nigrostriatal pathway remains relatively intact; currently, CERE-120 delivery solely to the putamen may be of limited therapeutic use. Furthermore, the fact that lack of retrograde transport to the nigra after intrastriatal injection to the human PD brain was unexpected on the basis of the pre-clinical data demonstrates an important limitation of current animal models of PD in terms of their applicability to the clinical situation.

The deterioration of dopaminergic neuronal terminals in the caudate-putamen, and the consequential loss of dopaminergic input to this region, supports the concept of targeting the striatum with PD therapeutics. Furthermore, this area is considered a more easily accessible surgical target than the substantia nigra pars compacta (SNpc). However, the Ceregene group has queried the standard approach of solely targeting putamenal dopaminergic terminals, in order to treat degeneration of the nigrostriatal pathway. Having observed poor distribution of CERE-120 after striatal delivery in patients compared with nonhuman primates (Ref. 109), they examined the safety and practicality of targeting the SNpc (Ref. 106). This study showed that intranigral delivery of CERE-120 was safe and well-tolerated in a rat PD model, and extrapolated appropriate nigral dosages of CERE-120 for future clinical studies. This group further found greater neuroprotection and a larger distribution area of NRTN protein in 6-OHDA-lesioned rats after intranigral than after striatal injection of CERE-120 (Ref. 108). They concluded that targeting the nigra, in addition to the

caudate-putamen, thus conferring simultaneous neurotrophic effects on both nigral dopaminergic cell bodies and their striatal terminals, would be more efficacious than striatal delivery alone (Ref. 108); they have adopted this strategy in their next clinical trial (Ref. 117). However, the targeting of the SNpc cannot be approached without caution. Three previous studies had reported significant weight loss in animals that had received a GDNF-expressing AAV2 vector into the SNpc (Refs 92, 96, 97). Thus, Bartus et al. compared an AAV2-GDNF reference dose against the range of CERE-120 doses being tested (Ref. 106). Weight loss was observed both in animals that received the AAV2-GDNF vector and those that had received the highest CERE-120 dose (Ref. 106). However, the authors attributed this weight loss to vector expression in mistargeted brain regions (Ref. 106). Such non-specific targeting must be kept minimal, if NTF treatments are to have a significant therapeutic benefit to risk ratio.

Ceregene's most recent open-label trial assessed the safety and efficacy of simultaneously targeting the SNpc and striatum. Patients received a total of four nigral doses and six putaminal doses of CERE-120 and were followed clinically for 2 years (Ref. 117). The intraputaminal dose was fourfold greater than previous trials, on the basis of safety data from the two preceding clinical trials in conjunction with dose-escalation studies in animals. Furthermore, the number of bolus injections was decreased from eight in the initial trial to three, and the infusion rate was increased. Analysis of data from the open-label, dose-escalation safety arm of this study in six patients reported no incidences of medical, immunological or psychiatric complications, for up to 24 months (Ref. 117). Due to the success of this safety trial, 51 patients were involved in the placebo-controlled double-blind phase IIb part of this study; half of these underwent sham surgery (Ref. 118). Although some of the key secondary endpoint measures did show a significant effect of treatment, this trial did not meet the predetermined endpoint of significant improvements in motor-off scores, compared with the sham surgery group (for review see Ref. 119). Also, significant motor-off benefits were observed in patients that had been diagnosed within 5 years prior to surgery, compared with those who were diagnosed at least 10 years prior (for review see Ref. 119). This finding highlights the necessity for earlier intervention approaches of NTF therapy in progressive neurological diseases such as PD, if significant clinical benefits are to be achieved.

CDNF

Two *in vivo* studies have so far examined the neurotrophic effects of CDNF via AAV2-mediated delivery in rats (Refs 54, 55). AAV2-CDNF conferred beneficial effects upon the nigrostriatal pathway to a certain extent in both studies (Refs 54, 55). Bäck et al. compared the ability of AAV2-CDNF to protect dopaminergic

neurons with that of AAV2-GDNF (Ref. 54). Intra-striatal injection of AAV2-CDNF in rats protected nigral dopamine neurons, and improved motor function, when administered 2 weeks before intra-striatal 6-OHDA, to a comparable extent to those of AAV2-GDNF (Ref. 54). Rats which received AAV2-CDNF did not display sprouting of dopaminergic neuronal terminals, in contrast to those that had received AAV2-GDNF. This is probably due to different distribution patterns of the proteins, as GDNF immunoreactivity was widespread, whereas CDNF was detected only within cells (Ref. 54). Intraneuronal confinement of CDNF protein following AAV-mediated delivery is probably the reason for the lack of neuronal sprouting, since such sprouting was found following infusion of CDNF protein into the striatal parenchyma (Ref. 53). This restricted expression of CDNF protein may explain the neuroprotective effects of AAV-CDNF being localised to the central part of the nigra, in contrast to the dopaminergic neuronal protection throughout the nigra that was conferred by AAV-GDNF (Ref. 54). Another study reported significant improvements in motor behaviour and rescue of nigral dopaminergic neurons and their striatal terminals, in rats that had received intra-striatal injection of AAV2-CDNF 6 weeks after intra-striatal 6-OHDA (Ref. 55). Some animals from that study were tested after 1 year and displayed long-term motor improvements (Ref. 55). Both of these studies indicate that the AAV2 system is a good candidate to test the delivery and effects of CDNF in other models of PD. A recent paper showed that combined administration of LV-CDNF and LV-MANF into the nigra, but not into the striatum, had significant protective effects on DA neuronal survival, and on amphetamine-induced rotational behaviour, in the 6-OHDA rat model of PD (Ref. 120). Furthermore, this study found that joint nigral overexpression of both factors had synergistic neuroprotective effects, suggesting that combined delivery of neurotrophic factors is a good avenue of exploration in the quest for an optimal neuroprotective therapy for PD.

GDF5

With regards to virus-mediated GDF5 delivery, research has been directed mainly at tendon repair, due to GDF5's properties as a bone morphogenetic protein. Two studies reported therapeutic improvements after AAV-mediated GDF5 delivery in rodent models of tendon injury (Refs 121, 122), proposing a nonsurgical approach for treatment of tendon injuries. Advances towards the use of viral vector-mediated GDF5 delivery to the brain for PD were made in two studies on 6-OHDA-lesioned rats (Refs 58, 123). The first transplanted GDF5-overexpressing embryonic rat dopaminergic neurons into the striatum of rats with striatal 6-OHDA lesions, and reported significant improvements in motor function in these rats, over those which had received control transplants (Ref. 123). This study provided proof-of-principle

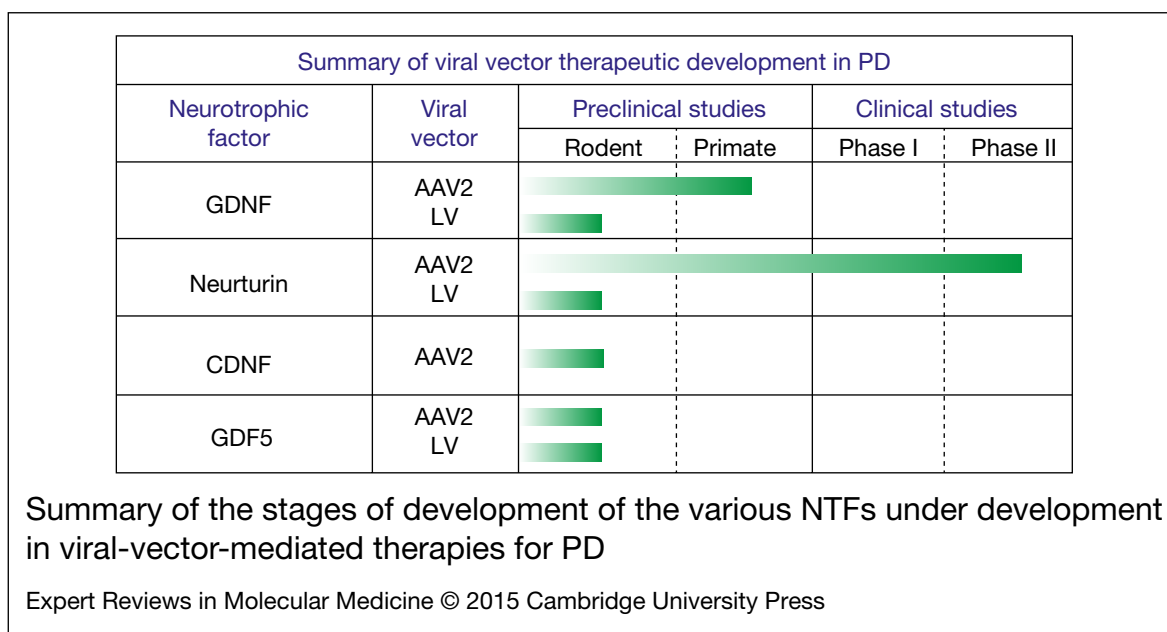


FIGURE 3

Summary of the stages of development of the various NTFs under development in viral-vector-mediated therapies for PD.

that GDF5 overexpression conferred therapeutic benefits, without any adverse effects, in a PD model. The second study reported improvements in motor function and increases in survival of transplanted dopaminergic neurons, following co-transplantation with a GDF5-overexpressing cell line, in rats with 6-OHDA lesions of the MFB or striatum (Ref. 58). Since viral vector-mediated GDF5 delivery has already been validated outside the nervous system, and since GDF5 overexpression by transplanted cells has shown efficacy in rat models of PD, it is timely for investigations of viral vector-driven GDF5 in PD models. We are currently investigating the efficacy of AAV- and LV-mediated delivery of GDF5 in 6-OHDA and AAV- α -syn rat models of PD. (For a summary of the current start-of-the-art and stage of development of viral-mediated NTFs for PD, see Fig. 3.)

Strategies for optimising NTF delivery in PD

Careful consideration must be given to the methodologies used to deliver optimised therapeutic agents in PD patients. The ideal delivery should be safe, efficient accurate and minimally invasive. Since NTFs do not cross the blood–brain barrier, this barrier must be bypassed via direct injection or infusion into the brain (for reviews see Refs 124, 66). This intracranial approach generally induces some trauma to the brain and has the risk of introducing infections. Alternative NTF delivery methods are being explored. For example, intranasal delivery, which has worked with other recombinant viral vectors (for review see Ref. 125), has been tested for GDF5 protein delivery to the brain (Ref. 126). Although the majority of the administered dose did not diffuse further than the olfactory epithelium, the nanomolar amount of GDF5 that

travelled to the midbrain was deemed to be a potentially therapeutic concentration (Ref. 126). Future studies may support the use of intranasal delivery for viral vectors delivering NTFs. Any such strategies must take into consideration the anatomical differences between the rodent and human nasal cavity. The olfactory epithelium covers a much larger proportion of the nasal cavities in rodents than in primates (Ref. 127), and thus it would be critical to test this route of delivery in nonhuman primates to determine the viability of this approach. Other propositions include the use of capsules designed through nanotechnology, thereby removing the need for viruses, or compression of vectors into nanoparticles to enable nuclear entry via nuclear pores (for review see Ref. 128). It will be some time before the first nanotechnologically-delivered NTF clinical trial is conducted, and the long-term effects of using these nanomaterials can be assessed.

A final point on target selection is the consideration of whether intraputamin vector treatments, such as AAV2-GDNF and CERE-120, are transported from the striatum to the substantia nigra in an anterograde or retrograde manner. Bartus et al. attributed the lack of CERE-120 transport to SNpc as failure of retrograde transport efficacy in the degenerating neurons (Ref. 109). It was on this basis that studies involving CERE-120 infusions into both striatum and substantia nigra were initiated (for review see Ref. 113). However, Bankiewicz and co-workers showed that nigral cell bodies can still receive intraputamin AAV2-GDNF via anterograde transport by nondopaminergic neurons of the striatonigral pathway (for review Ref. 129). Their rationale is that targeting the nigra with NTFs carries considerable health and

stereotactic risks, and that the intraputamenal CED approach alone is enough to therapeutically support the nigrostriatal neurons (Ref. 92). One risk with efficient transfer of NTF protein by anterograde trafficking is that areas additional to the target area may receive the therapeutic protein, which may cause off-target side-effects. If the upcoming AAV2-GDNF clinical trial yields more efficacious results than the CERE-120 clinical trial (Ref. 118), then it may be worth investigating whether CERE-120 could be more effectively delivered via intraputamenal CED. This could then be similarly applied to future viral vector-mediated delivery of other NTFs, such as CDNF and GDF5.

Conclusions

Gene therapy has contributed considerable advancements to the ongoing development of neuroprotective therapeutics for PD. Initial clinical trials involved administering GDNF protein, with mixed results (Refs 37, 38, 39, 40, 41, 42). The appearance of GDNF antibodies in some patients (Refs 41, 42) highlighted a need to approach NTF therapy differently. This led to testing of viral vector-mediated delivery of NTFs, with AAV2 vectors seemingly the most appropriate in the clinical scenario. Research on viral vector delivery of GDNF, and its optimisation via the use of CED, has led to the development of a protocol which will be implemented in an upcoming GDNF clinical trial (Ref. 91). Only minor benefits have been observed following viral vector-mediated NRTN delivery (Refs 115, 116, 117, 118), but trials incorporating optimised methodologies are ongoing. Regarding other dopaminergic NTFs, viral vector-delivered CDNF has been tested in rodents (Refs 54, 55) and GDF5 has been delivered using cell-based methods in rodents. It will be some time before these two NTFs are tested as potential PD therapeutics at the clinical level.

In summary, NTFs can play an integral part in the search for a viable treatment approach to PD. Current treatment options can only manage PD symptoms for a limited period of time. At the very minimum, NTFs could delay the onset of symptoms and slow disease progression. Since NTF proteins have a limited half-life in vivo and it is impractical to administer several applications of a NTF indefinitely, gene therapy presents huge potential for long-term and targeted treatment of PD.

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Corresponding author:
Dr Aideen Sullivan,
Department of Anatomy and Neuroscience,
University College Cork, Cork,
Ireland.
E-mail: a.sullivan@ucc.ie