

Review

Gene therapy for Parkinson's disease: recent achievements and remaining challenges

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Summary. Gene therapy is the use of nucleic acids as drugs. Thus, ways had to be developed to deliver this new generation of drugs to target tissues. Various viral and non-viral vectors have been engineered to carry potentially therapeutic nucleic acids into diseased organs or target cells. The brain offers a particular challenge for gene delivery to its constituent cells: it is encased by the skull, separated from the general circulation by the blood brain barrier, and made up of mostly non-dividing cells. The skull limits direct injection of vectors into the brain, the blood brain barrier inhibits the easy entry of vectors injected into the bloodstream, and post mitotic target cells restrict what type of vector can be used to deliver genes to the brain. We will discuss the main challenges faced by gene delivery to the brain, i.e. immune responses to the delivery vectors and therapeutic transgenes and length of duration of the therapy specifically as applied to Parkinson's disease. We will also discuss therapeutic strategies, which could be implemented to treat Parkinson's disease, and the models in which they have been tested.

Key words: Viral vectors, Adenovirus, Gene delivery, Neurodegenerative disorders, GDNF, Parkinson's disease.

Introduction

Parkinson's disease (PD) is a late-onset neurodegenerative disorder. It is characterised by the triad of bradykinesia, rigidity and resting tremor. Postural instability and asymmetry of motor signs in the limbs are also important features.

To make an accurate diagnosis of PD, different Parkinson-like syndromes, which may be due to drugs, cerebrovascular disease or other neurodegenerative causes need to be excluded. The gradual onset of the

disease and patients presenting a wide range of symptoms including pain, cognitive changes and depression, as well as symptoms complicated by the long term effects of drug treatment make diagnosis difficult and in over 20% of cases inaccurate (Hughes et al., 1992). The prevalence of PD in the general population is approximately 0.3% and rises dramatically over the age of 65 with almost 3% of people affected above that age. Mortality in PD is two to fivefold-increased (Berger et al., 2000).

Primarily, the pathology is due to progressive selective degeneration of dopaminergic neurones in the substantia nigra pars compacta. The presence of Lewy bodies, i.e., fibrillar neuronal inclusions, containing ubiquitin and α -synuclein, are the histological hallmark of the disease (Irizarry et al., 1998). Lewy bodies reported in individuals without clinical features of PD suggest a presymptomatic phase of the disease, which probably lasts 4 to 5 years as documented in a longitudinal positron emission tomography imaging study (Morrish et al., 1996). As a consequence of the degeneration of dopaminergic nigrostriatal fibres, striatal dopamine levels are reduced and the finely tuned circuitry of excitatory and inhibitory impulses in the basal ganglia is disturbed. Resulting subthalamic overactivity is considered to play a key role in the pathophysiology of PD (Blandini et al., 2000).

Current hypotheses include oxidative stress, mitochondrial dysfunction, immune mechanisms and excitotoxicity as possible mechanisms of nigro-striatal degeneration. Environmental factors also seem to contribute to the disease process and recently administration of a natural occurring pesticide, Rotenone, has been shown to reproduce the behavioural, anatomical, neurochemical and neuropathological features of PD in rats (Betarbet et al., 2000). A minority of mostly atypical cases is familial and several responsible mutations have been identified (Hattori et al., 2000).

To date therapeutic strategies are mainly symptomatic. Dopamine replacement with its precursor levodopa in combination with a decarboxylase inhibitor continues to be the most effective treatment decreasing

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the mortality from PD. It provides adequate control of symptoms for many years. Major problems associated with its long term use are disabling dyskinesias and motor fluctuations. After 10 years duration a study examining young-onset Parkinson's disease showed that almost all patients were experiencing severe motor fluctuations and dyskinesias (Schrag et al., 1998). How much these are a result of natural disease progression or levodopa exerting a neurotoxic effect has not yet been determined (Agid, 1998). The pulsatile administration of short acting dopaminergic drugs, primarily levodopa, is now thought to be an important factor in developing motor complications. The rate of developing dyskinesias can be significantly slowed by initial treatment with the dopamine agonist Ropinirole alone (Rascol et al., 2000). Levodopa treatment in combination with a catechol-O-methyltransferase inhibitor has been shown to even out motor oscillations in fluctuating Parkinson's disease, by increasing the half-life and bioavailability of levodopa (Piccini et al., 2000).

Surgical treatments for PD lost their impact after the introduction of levodopa; only in the 1990's have neurosurgical approaches regained their role for advanced complicated disease with a greater understanding of the pathophysiology, more refined imaging and electrophysiological localisation-techniques. The targets are the ventral intermediate nucleus of the thalamus, to alleviate drug refractory tremor, the globus pallidus internus, achieving good results mainly for levodopa associated dyskinesias and the most promising one, the subthalamic nucleus (STN). Targeting the STN leads to a striking reduction in all the signs and symptoms of PD, which is sustained for several years. Levodopa dosage is reduced by 50% in these patients, which further diminishes dyskinesias (Limousin et al., 1998). Inactivation by high frequency deep brain stimulation is now favoured over ablating these areas, mainly because it can be performed bilaterally quite safely and is reversible. Serious but rare complications are haematoma and infection. In about a quarter of patients hypophonia and eyelid apraxia occur.

Another treatment approach, which can restore motor function, is human fetal mesencephalic tissue transplantation. Reports from different groups stated clinical improvement lasting for three to four years. In March of this year results of the first prospective double-blind, sham-surgery controlled clinical trial were published. Double-blind assessments were completed for twelve months and a small clinical benefit on standardized rating scales while off medication in the 60 years and younger age group could be demonstrated. This improvement persisted for three years, which is the end of the reported follow up period. There were no significant improvements in the older patients in the transplanted group. After improvement in the first year, dystonia and dyskinesias occurred in 15 percent of the transplanted patients, in spite of reducing or even discontinuing the dose of levodopa. The main conclusion for this study was that some clinical benefit was

achieved in younger but not in older transplanted patients (Freed et al., 2001).

In depth reviews of current insights into the pathophysiology of PD - in particular associated with levodopa therapy - are compiled in: Basal Ganglia, Parkinson's disease and levodopa therapy: a supplement to Trends in Neurosciences (Olanow et al., 2000).

In view of what we discussed above finding new treatments for neurodegenerative disorders, like PD, has been a challenge for clinicians and scientists during the past twenty years. Gene therapy, which aims to deliver nucleic acids as drugs, constitutes an important new treatment modality for this devastating disorder.

Neurological gene therapy: The main challenges

While the number of patients entered into clinical trials of gene therapy has continued to increase over the last years, the implementation of the treatment of brain diseases using gene therapy strategies still poses formidable practical challenges. In consequence, clinical trials of neurological gene therapy are still under-represented among the active clinical trials, even though new neurological therapies are certainly needed. Major neurological diseases serious enough to merit the targeted development of new therapies are various malignant brain tumours and selected progressive neurodegenerative diseases, such as PD.

The main challenges holding back the widespread clinical implementation of neurological gene therapy are the technical limitations of current transgene delivery systems, i.e., the gene transfer vectors (Verma and Somia, 1997). Generally, short term expression of the potentially therapeutic transgenes, coupled to the instability of vectors in the presence of the inflammatory and immune responses directed against the vectors and/or transgenes, reduce the efficiency of delivered therapeutic transgenes (Wood et al., 1996; Thomas et al., 2001a). Factors affecting vector stability in target cells/tissues, remain to be identified. Whether integration of the vector-delivered DNA into the genome of target cells is needed for stable long term transgene expression remains unknown. In many cases where integration into the genome occurs, transgene expression encoded by 'classical' murine retroviral vectors still becomes silenced over time. In contrast, new data using 'gutless' adenoviruses and lentiviral vectors appear to indicate that long term expression can be achieved both in the presence and absence of integration (Blomer et al., 1997; Verma and Somia, 1997; Morral et al., 1998; Thomas et al., 2000). Mechanisms underlying instability of long term transgene expression are likely to be vector and/or transgene specific and remain to be determined. The injection of adenoviruses into the brain induces inflammatory and immune responses, and can also induce direct cytotoxicity. Inflammatory responses occur immediately following virus injection. They include a rapid release of cytokines (e.g. IL-1, IL-6, or TNF α), and rapid recruitment of macrophage/monocytes/

microglia, as well as CD8+ cells, and upregulation of MHC I and II expression (Wood et al., 1996; Cartmell et al., 1999; Thomas et al., 2001a). Direct cytotoxicity, can include direct neuronal and glial cytotoxicity. Inflammation and direct cytotoxicity occur very early upon viral vector injection and are dose dependent. They are not seen below 1×10^7 i.u., and become very severe at concentrations above 1×10^8 i.u. (Thomas et al., 2001a). Immune responses also occur after injection of virus into the brain. A humoral response can be detected, which is non-neutralising. If the injection is confined to the brain parenchyma, there is no priming of a cytotoxic T-cell response. If, however, the injection is made into the brain ventricles, a cytotoxic T-cell response, and a neutralising B-cell humoral response is elicited. A peripheral injection of adenoviruses will prime the immune system, and leads to the elimination of transgene expression from first generation viral vectors, but not, novel high capacity adenoviral vectors (Thomas et al., 2000). However, in some experimental paradigms, inflammatory and immune cells have been shown to be beneficial for neuronal survival to injury: thus, the therapeutic implications of inflammatory and immune processes for long term transgene expression need to be assessed (Dewey et al., 1999; Thomas et al., 2000).

New vector developments

The aim of gene therapy is to introduce therapeutic genes into cells or tissues, thus leading to efficient and stable expression of the recombinant protein minimizing any adverse effects. Viruses can easily enter into cells and display their genetic material in the nucleus and therefore are much more efficient vectors than non-viral systems. Wild type viruses, however, need to be engineered to be rendered non deleterious for the patient and to carry and express the foreign DNA sequences (Fig. 1). The main modifications are the elimination of viral replication and the consequent cytotoxic/cytolytic properties and the introduction of the transgene(s) into the viral genome without affecting other viral structures and properties (such as packaging, cell entry pathway, integration). Many viruses have been tested as vectors and the most commonly used are recombinant retrovirus, adenovirus (Ad), herpes simplex virus (HSV) and adeno-associated virus (AAV) (Castro et al., 2000; Stone et al., 2000; Lowenstein et al., 2001).

Recombinant retroviruses have been widely studied and the majority of clinical trials have indeed used vectors based on the Moloney murine leukaemia virus (Mo-MLV) (Clinical trials update, 1999). These RNA viruses are useful because their genome integrates into the host chromosomes and therefore are more stable than viruses whose sequences remain episomal. However, this property has the reverse effect of potentially inducing insertional mutations. The drawback of retroviral vectors, which is a major obstacle to brain gene transfer, is their inability to transduce non-dividing cells (Roe et al., 1993).

On the other hand, lentiviruses are complex retroviruses, which have the capacity of infecting quiescent cells such as neurones, and have therefore recently been developed as vectors (Naldini et al., 1996a). Human immunodeficiency virus (HIV)-1-derived vectors were attained by transfection of a three-plasmid expression system (Naldini et al., 1996a) and have been the most studied. This system, by pseudotyping the virion particles with the amphotropic envelope of MLV or the G glycoprotein of vesicular stomatitis virus (VSV-G), extended the restricted HIV infection spectrum ($CD4^+$ T cells) to other cell types. Using these vectors, Naldini et al. have reported production at high titres, efficient transduction *in vitro* and *in vivo*, associated with transgene integration into the host genome, and long-term expression in rat brains without signs of pathology (Naldini et al., 1996b). Similar results have been obtained after administration of these lentiviral vectors into retina, liver or muscle (Kafri et al., 1997; Miyoshi et al., 1997). With better fundamental knowledge of the lentiviral structure and biology, new systems (including deletions, self-inactivating vectors or new packaging cell lines) have been developed, which yield higher titres and safer preparations (Zufferey et al., 1997, 1998; Dull et al., 1998; Miyoshi et al., 1998; Kafri et al., 1999; Klages et al., 2000; Wu et al., 2000). HIV-derived lentivirus vectors, however, still raise some safety concerns and need to be checked for several parameters (such as formation of replication competent virus by homologous recombination, integration into host genome, recombination with endogenous human retroviruses and the spread by wild type virus to untransduced cells) before one can consider using them in clinical trials.

Considering biosafety issues, other vectors using bovine, simian (SIV) or feline (FIV) immunodeficiency virus (Poeschla et al., 1998; Curran et al., 1999; Johnston et al., 1999), sheep lentivirus Visna (Berkowitz et al., 2001), equine infection anaemia virus (EIAV) (Olsen, 1998) or caprine arthritis and encephalitis virus (CAEV) (Mselli-Lakhal et al., 1998, 2000) have recently been developed.

First generation recombinant adenovirus (rAd) vectors have been widely described as powerful vectors and present several interesting features such as production of high titre preparations, broad spectrum of cell type infectivity including dividing and non-dividing cells, and high transduction efficiency both *in vitro* and *in vivo* (Danthinne and Imperiale, 2000). These vectors have however the drawbacks of a limited transgene size (8 kb) and the induction of inflammation and immune responses in the host leading to rapid transgene clearance *in vivo* (Yang et al., 1994a,b). This phenomenon has been identified in many organs, but does not affect the brain, since a single rAd injection into the rat striatum leads to stable long-term (at least 1 year) transgene expression (Thomas et al., 2000). The situation is different however if the animal has been previously exposed to Ad, which is the case in a large

majority of humans; then transduced brain cells are also eliminated rapidly by a specific anti-Ad immune response (Thomas et al., 2001b).

To circumvent these drawbacks, high capacity (HC) Ad vectors, also called helper-dependent (HD) or "gutless" Ad vectors, have been developed (Clemens et al., 1996; Haecker et al., 1996; Kochanek et al., 1996; Parks et al., 1996; Umaña et al., 2001). These vectors have been deleted for nearly all their viral DNA sequences (only viral inverted terminal repeats (ITRs) and packaging signal remain) and allow a cloning capacity of ~28 kb. However, they require the presence of helper virus (providing in *trans* all sequences necessary for viral replication, packaging and encapsidation) and special packaging cell lines to be

produced (Kochanek et al., 1996; Parks et al., 1996; Umaña et al., 2001). Many studies reported the efficiency of these HC-Ad vectors illustrated by reduced immunogenicity and prolonged transgene expression in different *in vivo* models (Chen et al., 1997; Morsy et al., 1998; Schiedner et al., 1998; Morral et al., 1999). In the brain, work from our group has shown the persistence of HC-Ad-mediated transgene expression even consecutively to peripheral Ad-priming (Thomas et al., 2000, 2001b). These vectors are very promising, although their production still requires some technical improvements to achieve higher titres and lower contamination by helper virus and their biosafety needs to be defined before starting clinical trials.

HSV type 1 has been exploited for gene transfer in

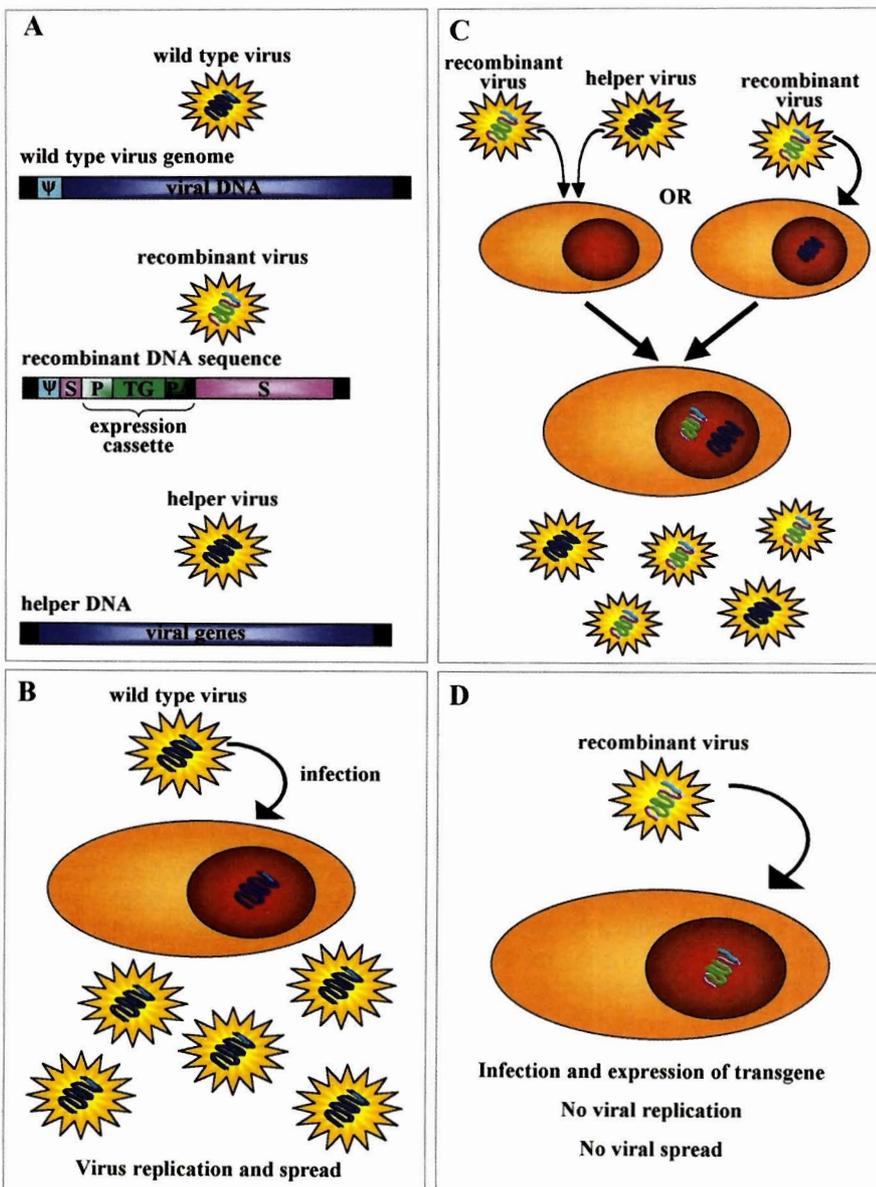


Fig. 1. Schematic representation of gene therapy strategy using viral vectors. Viral gene therapy approaches use the capacity of viruses to enter the cell and deliver their DNA content into the nucleus. However, these strategies are beneficial only if the wild type virus is rendered deficient for replication and the DNA sequence from the gene of interest introduced into the viral genome. **A.** Schematic representation of composition of wild type and modified viruses. The wild type viral genome contains all viral genes and the packaging signal (ψ). This virus is modified to create a recombinant virus missing most viral genes but containing the transgene expression cassette, i.e., promoter (P), transgene (TG), polyA tail (PA), the packaging signal (ψ) and stuffer DNA (S) to reach a size allowing packaging. This recombinant virus alone is not able to replicate and needs the presence in *trans* of viral DNA to produce recombinant virions. Therefore a helper virus is constructed containing all essential viral genes but lacking the packaging signal. This virus alone is not able to package its DNA. **B.** Schematic representation of infection with a wild type (WT) virus. The virus enters the permissive cell and the genetic content (all the viral genes are present in the WT virus) is released into the host nucleus. The replication and transcription of viral DNA are initiated, viral proteins are produced, the genetic material is packaged and new virions are released. **C.** *In vitro* production of recombinant viral vectors. The recombinant virus, lacking viral genes required for replication and production of virions, but containing the transgene and the packaging signal, is not able to produce any virions if administered alone into the cells. However, if this virus is co-administered with a helper virus containing the viral genes in *trans* or if the host cell has been transfected with the viral sequences, recombinant and helper virus will be produced. Recombinant viral vector can then be amplified and purified. **D.** *In vitro* or *in vivo* transduction with recombinant viral vector. In the situation where there are no viral genes in *trans*, the transgene will be delivered into the host cell and the recombinant protein will be produced, but no further virion will be produced, rendering this system very safe.

different *in vitro* and *in vivo* models. Despite its wide transduction spectrum, it has been especially used in the central nervous system because of its ability to establish latency in neurones. Its genome structure allows large inserts (up to 30 kb). HSV-1 vectors expressing stably for up to 18 months in dorsal root ganglia and brain stem neurones have been produced (Carpenter and Stevens, 1996). Whether similar vectors could also be used for long-term transgene expression in forebrain neurons of the striatum and/or neocortex remains to be determined. These vectors have been used in different tumour models especially using the HSV-thymidine kinase (TK) gene which displays cytopathic effects in transfected cancer cells (review by Latchman, 2000). Inflammation, immune response and toxicity are the main concerns that limit the use of these vectors to treat chronic neurodegenerative disorders in humans.

AAV are small viruses, which are able to infect a wide range of cells including dividing and resting cells. No pathogenicity has been associated with the infection. AAVs have the capacity of establishing latency in the host cell and to integrate their DNA into a specific locus of the host genome (chromosome 19 in humans). The rescue phase requires infection by a helper virus such as Ad or HSV. AAV-derived vectors are promising, however, production of pure rAAV preparations at high titres is cumbersome. AAV vectors seem to lose the ability of integrating their DNA into a specific site and the viral DNA can also remain episomal. But overall, the main drawback is the limited capacity for foreign sequences (~4-5 kb). However, many groups are working on improvements on these vectors, which have produced very limited evidence of an immune response or toxicity when administered *in vivo* (Xiao et al., 2000). Circulating specific antibodies have been identified, however their effects have not been determined yet and need to be addressed, especially in the context of vector re-administration. Recently, dimer vectors have been characterised accepting transgenes twice as big. (Haberman et al., 2000; Nakai et al., 2000; Yan et al.,

2000).

Among the viral vectors described previously, none is, at present, ideal for human gene therapy, the advantages and drawbacks of each virus are summarized in table 1. With the aim of combining beneficial properties, chimeric vectors have been developed such as AAV/Ad (Recchia et al., 1999) or Ad/retrovirus (Reynolds et al., 1999), however these developments are in their infancy. Other authors have concentrated their efforts on the optimisation of non-viral vectors, which offer more safety but are less efficient.

For some disorders, gene transfer needs to address a specific type of cell. This targeting can be achieved by two different ways: one is to confine the expression of the transgene and this can be done by choosing an appropriate promoter, the other is to modify the tropism of the virus, often done by engineering the capsid (Russell and Cosset, 1999; Krasnykh et al., 2000).

Finally, it may be required that transgene expression is restricted in time and therefore inducible systems have been developed where transgene expression can be switched "on" or "off" (Agha-Mohammadi and Lotze, 2000). The most common system involves the tetracycline activator and responsive elements and results in activation or inactivation of the transgene depending on the presence or absence of tetracycline or doxycycline (Ghersa et al., 1998; Harding et al., 1998; Kafri et al., 2000; Smith-Arica et al., 2000).

Preclinical models for the implementation of new treatment strategies for Parkinson's disease

Non-human models of Parkinson's disease are crucial for understanding the causes and progression of neurodegeneration and for developing efficient new therapies. The standard animal models currently used are the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)-treated primate and the 6-Hydroxydopamine (6-OHDA)-treated rodent (Gerlach and Riederer, 1996; Bezard et al., 1998; Tolwani et al., 1999; Luquin, 2000).

Table 1. Advantages and drawbacks of viral vectors for gene therapy.

VIRUS	INSERT SIZE	ADVANTAGES	DRAWBACKS
Retrovirus	8 kb	Broad cell tropism, Integration into host genome, no toxic effects	Require cell division, risk of insertional mutagenesis, degradation by complement, risk of recombination with human endogenous retrovirus
Lentivirus	8 kb	Infection of dividing and non-dividing cells, integration into host genome	Risk of insertional mutagenesis, risk of seroconversion
Adenovirus	8 kb	Broad cell tropism, infection of dividing and non-dividing cells, relatively easy to produce at high titres	Transient expression inflammatory and immune responses
HC-Ad	28 kb	Broad cell tropism, infection of dividing and non-dividing cells, no inflammatory and cellular immune responses	
AAV	5 kb, 10 kb *	Broad cell tropism, infection of dividing and non-dividing cells, integration into host genome (specific locus)	Difficult to produce pure preparations at high titre, discrete immune response
HSV	30 kb	Broad cell tropism, latency in neurones, very stable	Highly toxic

*size can double if concatamers are used.

These models are based on toxic destruction of dopaminergic brain cells (and some other types), and usually employ young, otherwise healthy animals.

MPTP was discovered as a contaminant in batches of illegally synthesised meperidine, and was found to be responsible for the parkinsonian symptoms of individuals who ingested the MPTP-contaminated narcotics. Systemic administration of MPTP to primates causes severe motor abnormalities, producing the majority of cardinal motor symptoms of PD, with the exception of tremor. These effects were attributed to a loss of dopaminergic neurones, which follows MPTP treatment. The neurotoxic effect of MPTP is believed to be related to its metabolism by MAO-B in glial cells to produce MPDP⁺ and subsequently MPP⁺, which then accumulates within dopaminergic neurones. Presumably, the specificity of MPTP for dopaminergic (DA) neurones is related to its metabolites being taken up into DA neurones by way of the uptake transporter. It is believed that ultimately the toxicity of MPP⁺ is due to uptake into mitochondria, and subsequent inhibition of the respiratory chain.

The chemical structure of 6-OHDA is highly analogous to that of the catecholaminergic neurotransmitters (i.e. dopamine, noradrenaline). Thus, the membrane uptake systems of catecholaminergic neurones, which usually transport their transmitters back, can take up 6-OHDA into the cell where the substance then accumulates. Since 6-OHDA is very electro-active, it will rapidly oxidise, leading to several cytotoxic compounds, such as free radicals and hydrogen peroxide, which can damage the neurones by affecting proteins, membrane lipids, and DNA. Once 6-OHDA is taken up into catecholaminergic cells, and metabolised into its cytotoxic metabolites, these substances lead to cell destruction, mainly by destroying the neuronal membrane. Since 6-OHDA does not efficiently cross the blood-brain barrier, the compound must be administered into the brain in order to destroy catecholaminergic cells, or under certain conditions, only dopaminergic cells (Schwartz and Huston, 1996).

The 6-OHDA model of Parkinson's disease is considered particularly convenient, since it is "relatively" easy and inexpensive to generate and use. The severity of the 6-OHDA lesion and its behavioural effects are not only affected by the total dose of the neurotoxin injected, but also by the site of neurotoxin administration (i.e. substantia nigra, medial forebrain bundle (MFB) or striatum). Injection of 6-OHDA into either the substantia nigra or the MFB produces a complete lesion, whereas intrastriatal administration of 6-OHDA produces lesions ranging from mild to severe (Kirik et al., 1998). The first two lesion types are acute with clear signs of DA neurodegeneration appearing rapidly within 24 hours. Initially, striatal administration of 6-OHDA induces direct toxic damage to DA innervation close to the injection site, which is followed by a progressive loss of DA neurones in the substantia nigra (Sauer and Oertel, 1994, Kirik et al., 1998).

Several 6-OHDA-rat models with complete or partial lesions of the substantia nigra have been developed (Ungerstedt and Arbuthnott, 1970; Sauer and Oertel, 1994; Lee et al., 1996; Kirik et al., 1998). In the complete lesion models, the animals received an unilateral injection of 6-OHDA into the ascending mesostriatal pathway originating from the DA neurones of the substantia nigra and the one originating near the ventral tegmental area. This causes a total lesion of DA neurones in the substantia nigra, and a complete denervation of the ipsilateral striatum from its DA afferents (Ungerstedt and Arbuthnott, 1970). In the partial lesion model, the animals are given unilateral striatal injection of 6-OHDA. This causes a partial lesion of the DA neurones in the substantia nigra and partially denervates the ipsilateral striatum from its DA afferents. This degeneration takes place progressively (Sauer and Oertel, 1994). The 6-OHDA lesion of nigral neurones in rats results in rotational behaviour upon administration of dopaminergic agents (i.e. amphetamine, apomorphine, L-dopa).

Non-human primate models of PD are also essential, in understanding the pathophysiology of the disease and in developing and testing new therapies, such as gene therapy and cell transplantation. The non-human primate brain is more similar to the human brain than brains from any other species. It is increasingly apparent that many of the neurochemical changes associated with nigrostriatal dopaminergic loss that are observed in humans and monkeys cannot be demonstrated in rodents. Moreover, because the non-human primate motor repertoire closely resembles that of humans, it is easy to distinguish signs of parkinsonism in MPTP-treated monkeys, and to interpret the clinical significance of effective clinical interventions in this model (Bezard et al., 1997; Eberling et al., 1998; Ghorayeb et al., 2000). The MPTP-treated non-human primate is currently the most accurate model of PD in terms of neurochemical anatomy and response to symptomatic and restorative treatment (Gerlach and Riederer, 1996; Bezard et al., 1998; Tolwani et al., 1999; Luquin, 2000). Unfortunately, relatively few centers have the resources to use primates and many research facilities lack primate facilities. In addition to primate housing and routine care, specialised resources are essential for many types of studies, including neuroimaging capacities, expertise in neurosurgery, generation of Parkinson's models, and clinical evaluation of primates.

Because these animal models mimic some key features of PD, they are useful to evaluate novel therapeutic strategies. The predictive value of these models has been repeatedly validated for dopaminergic agents: anti-parkinsonian drugs that appropriately modify motor behaviour in these models have characteristically proven symptomatically effective for patients (Grunblatt et al., 2000). However, although these models play an important role in the study of PD, they are far from adequate. They do not reproduce the cause of PD, the progressive neurodegeneration, or the

destruction of non-dopaminergic cells (Gerlach and Riederer, 1996; Bezdard et al., 1998; Tolwani et al., 1999; Luquin, 2000). The validity of these models to predict the efficacy of neuroprotective or neurorestorative strategies has yet to be demonstrated. Indeed, lesioning in either model is generally done acutely, producing rapid severe injury to the nigral dopaminergic neurones, which clearly differs from the slowly progressive nature of the degenerative process in PD. Conceivably, such animal models might have a higher predictability index when used for testing neurorestorative strategies, at which point earlier steps in the pathogenic cascade might not be as critically relevant in the evaluation of neuroprotective approaches (Mouradian and Chase, 1997).

A spectrum of novel models of PD is necessary to study all the aspects of the disease and new treatment outcomes. Transgenic animals represent the best hope for producing animal models that faithfully represent the pathogenesis and progressive neurodegeneration of PD. Recently a *Drosophila* model of PD has been developed by expressing normal and mutant forms of alpha-synuclein. This model recapitulates essential features of PD such as locomotor dysfunction, adult-onset loss of dopaminergic neurons and filamentous intraneuronal inclusion containing alpha-synuclein (Feany and Bender, 2000). Moreover, transgenic mice expressing wild-type human alpha-synuclein were recently generated. Neuronal expression of human alpha-synuclein resulted in progressive accumulation of alpha-synuclein and ubiquitin-immunoreactive inclusions in neurones in the neocortex, hippocampus, and substantia nigra. Ultrastructural analysis revealed both electron-dense intranuclear deposits and cytoplasmic inclusions. These alterations were associated with loss of dopaminergic terminals in the basal ganglia and with motor impairments suggesting that accumulation of wild-type alpha-synuclein may play a causal role in PD and related conditions (Masliah et al., 2000; Sommer et al., 2000; van der Putten et al., 2000). These mouse models provide means to address key aspects of PD and also test new therapeutic strategies.

Gene therapy strategies for Parkinson's Disease

Gene therapy for PD is very promising, two main avenues have been explored to evaluate this approach: 1) neuroprotection and/or regeneration of dopaminergic neurones and 2) biochemical restoration. The former is intended to protect or recover dopaminergic neurones from the underlying degenerative process, by utilizing growth factors such as glial derived neurotrophic factor (GDNF) and other members of this family of neurotrophic factors. The second aims to transfer genes coding critical enzymes for the biosynthesis of dopamine, such as Tyrosine Hydroxylase (TH), the rate limiting enzyme in the biosynthesis of dopamine. Viral vectors have been successfully used for the delivery of therapeutic genes in *in vivo* and *in vitro* models of PD.

These include: Adenovirus (Ad), Adeno-associated vectors (AAV), Herpes simplex virus (HSV) and more recently Lentivirus (Lt).

Using adenoviral vectors, Choi Lundberg et al. (1997, 1998), have demonstrated that intracranial injection, either dorsal to the substantia nigra or at the level of the dorsal striatum, of an adenovirus encoding GDNF results in a 67% or 40% protection, respectively, of fluorogold (FG)-labelled dopaminergic neurons from the toxic insult with 6-OHDA. In addition to this effect, it was also shown, that amphetamine-induced rotation and the asymmetry in forelimb use were prevented when the AdGDNF was delivered into the denervated rat striatum (Choi Lundberg et al., 1997). Using the striatal paradigm, Bilang-Bleuel et al. (1997) obtained similar results. A more recent study in aged rats confirmed these anatomical and behavioural findings when the AdGDNF was injected into the denervated striatum (Connor et al., 1999). However, in contrast to young rats (Choi Lundberg et al., 1997), injection of AdGDNF into the substantia nigra pars compacta (SNpc) of aged rats protected dopaminergic neurons to a lower extent from the challenge with 6-OHDA. No significant effect in the recovery of behavioural deficits was detected (Connor et al., 1999). By using a recombinant adenovirus to transfer and express the TH gene into 6-OHDA denervated striatum, it was possible to decrease the turning behaviour of treated rats (Horellou et al., 1994). A further improvement of this strategy was the introduction of a doxycycline (Dox) regulatable tet-off system into the adenoviral expression cassette. This approach resulted in the increase of striatal levels of L-dopa when the TH expression was allowed in absence of Dox and with concomitant exogenous administration of the cofactor BH4 (necessary for the hydroxylation of tyrosine and decreased in 6-OHDA denervated rats). In contrast, TH expression was abolished in denervated rats, after administration of Dox in the drinking water. Furthermore, no effect was observed in the striatal levels of L-dopa, even in the presence of the cofactor BH4, under this repressive paradigm. Hence, these regulatable vectors represent a promising tool in the future clinical implementation of gene therapy, to avoid dyskinesias and the motor fluctuations that result as consequence of chronic L-dopa treatment in patients with PD (Corti et al., 1999). A synergistic antiapoptotic and neurorestorative strategy using an adenoviral vector encoding an inhibitor of caspases, the X-chromosome linked inhibitor of apoptosis (XIAP), and another adenovirus encoding GDNF, proved to be effective for protection of dopaminergic neurones and restoration of nigrostriatal terminals after toxic insult with MPTP. Interestingly, despite efficient expression of GDNF in the striatum and SNpc this molecule alone was unable to protect DA neurons from degeneration, but was able to restore levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). In contrast, Ad-XIAP was able to protect dopaminergic neurones in the absence of striatal terminals restoration. Only when a combined

treatment with Ad-XIAP and Ad-GDNF was administered, was protection against cell death and depletion of striatal DA, DOPAC, HVA achieved (Eberhardt et al., 2000). Recently, it was proposed that an adenovirus encoding sonic hedgehog (shh) is able to increase the survival of dopaminergic neurons in ventral mesencephalic cultures and protect 54% of FG labelled DA neurones against the neurotoxic insult with 6-OHDA compared to 33% in β -galactosidase (Gal) treated rats (Hurtado-Lorenzo et al., 2001).

Using the GDNF and TH therapeutic approaches, recombinant AAVs have also been used. A recombinant adeno-associated vector expressing GDNF under the control of a modified version of human cytomegalovirus CMV promoter was able to generate significant protection of fluorogold labelled dopaminergic neurons when the vector was injected into the substantia nigra three weeks before induction of degeneration with 6-OHDA (Mandel et al., 1997). The same authors recently demonstrated that this vector encoding GDNF, but injected after OHDA increased survival of dopaminergic neurons (Mandel et al., 1999). Behavioural tests were not performed in either of these studies. Using a biochemical restoration approach, it has been demonstrated that AAV-mediated co-expression of human TH together with human GTP-cyclohydrolase I (rate-limiting enzyme in the production of cofactor BH4) gene, in the striatum of 6-OHDA lesioned rats, resulted in the striatal production of L-dopa. However, despite the biosynthesis of this precursor, reduction of apomorphine induced rotational behaviour was not achieved (Mandel et al., 1998). It was also shown that the transfer of an AAV encoding TH into a 6-OHDA denervated striatum was sufficient to induce behavioural recovery of the treated rats (Kaplitt et al., 1994). Similar results have been obtained after striatal gene transfer of TH and human GTP-cyclohydrolase I in dopamine deficient mice lacking TH in dopaminergic neurones. The expression of both enzymes resulted in recovery of feeding behaviour in these mutants, which are characterized by hypoactivity and aphagia (Szczepka et al., 1999).

In the past few years, HSV vectors have also been used to transfer therapeutic genes into the brain of parkinsonian rats. An early study reported the increase of both TH activity and levels of dopamine in the denervated striatum of 6-OHDA lesioned rats when a HSV encoding the TH gene was injected into the striatum. Furthermore, a decrease of 64% in the apomorphine induced rotational behaviour was obtained. This effect was sustained for one year after gene transfer (Doring et al., 1994). Several lines of evidence indicate that apoptosis can be proposed as one of the mechanisms contributing to neurodegeneration in PD (Michel et al., 1999) and as the main mechanism of action of 6-OHDA (Walkinshaw and Waters, 1994; He et al., 2000). In addition, features of apoptosis have been found in post-mortem studies of human parkinsonian brains (Tomkins et al., 1997). Based on these findings another strategy for

treatment of PD has been the utilization of antiapoptotic gene therapy. By using a HSV expressing the antiapoptotic proto-oncogene bcl-2, it was recently demonstrated that direct injection of this gene into the substantia nigra of Sprague-Dawley rats, resulted in a 50% protection of the TH⁺ immunoreactive and fluorogold-labelled neurones in the substantia nigra (Masanobu et al., 1999; Yamada et al., 1999).

HIV-1 based lentiviral vectors have recently been introduced for gene transfer into the CNS. A Lentivirus vector encoding Neuroblastin/Artemin, a new member of the GDNF family, under the control of the human CMV promoter, was able to enhance the survival of TH⁺ cells in cultures of ventral mesencephalon and organotypic slice cultures by 60-70%. In addition, injection of this vector into the rat striatum resulted in an 80-90% protection of fluorogold labelled TH⁺ cells against 6-OHDA induced lesions (Rosenblad et al., 2000). One of the most outstanding results using lentiviral (Lt) vectors for the delivery of GDNF, was recently published by Kordower et al. (2000). In this study the effectiveness of Lt-GDNF in reversing the cellular and behavioural changes associated with nigrostriatal degeneration in non-human primates was evaluated. Two experimental groups were established. In the first group, aged monkeys (25 years-old) were injected intracranially with Lt-GDNF or Lt- β -Gal at the level of the caudate nucleus and the putamen. This treatment resulted in a 27% increase in the ¹⁸F-fluorodopa uptake and an 85% increase in the numbers of TH immunoreactive (TH-ir) nigral neurones. In the second group Lt-GDNF was administered in young monkeys 1 week after induction of nigrostriatal degeneration mediated by intracarotid administration of MPTP. The simultaneous intranigral and intrastriatal delivery of this vector resulted not only in complete protection of dopaminergic neurons but also in an additional 32% increase in the number of TH-ir neurones and 11% increase in TH-ir density. In contrast, control animals treated with Lt- β -Gal showed a decrease of 89% and 81% in the number and the density of TH-ir neurones respectively. Lt-GDNF treatment also increased the volume of TH-ir neurones (44.3% relative to contralateral site or β -Gal) and the levels of TH mRNA (41% relative to contralateral site of β -Gal). Interestingly, the treatment with Lt-GDNF also increased the ¹⁸F-fluorodopa uptake by 300%, improved the performance in the operant hand reach task and enhanced the striatal TH immuno-reactivity. These results suggest that Lt-GDNF treatment not only protected TH-ir neurones but also preserved dopaminergic terminals (Kordower et al., 2000).

Safety concerns regarding the use of replication deficient lentiviral vectors include the possible reversion to replicative competent forms. However, a vector with enhanced safety properties, which is multiply attenuated and self-inactivating, was successfully used for the treatment of parkinsonian animals (Deglon et al., 2000). In this study a lentiviral vector carrying the

GDNF gene under the control of the PGK promoter, was able to protect 51% of dopaminergic neurones after mechanical axotomy of the medial forebrain bundle. Further improvement of these lentiviral vectors involved the addition of the woodchuck hepatitis virus posttranslational fragment to the DNA sequence of the gene of interest. This modification resulted in a significant increase in the levels of gene expression (Deglon et al., 2000; Rosenblad et al., 2000), presumably by enhancing the stability of RNA

transcripts or by augmentation of their exportation to the cytoplasm (Zufferey et al., 1999).

Today the utilization of viral vectors encoding neurotrophic factors, antiapoptotic genes and enzymes involved in dopamine biosynthesis, seems promising for the treatment of PD. However, the efficacy of these therapeutic approaches in parkinsonian patients can only be determined in clinical trials and after several safety and functional aspects of the use of viral vectors have been stringently evaluated.

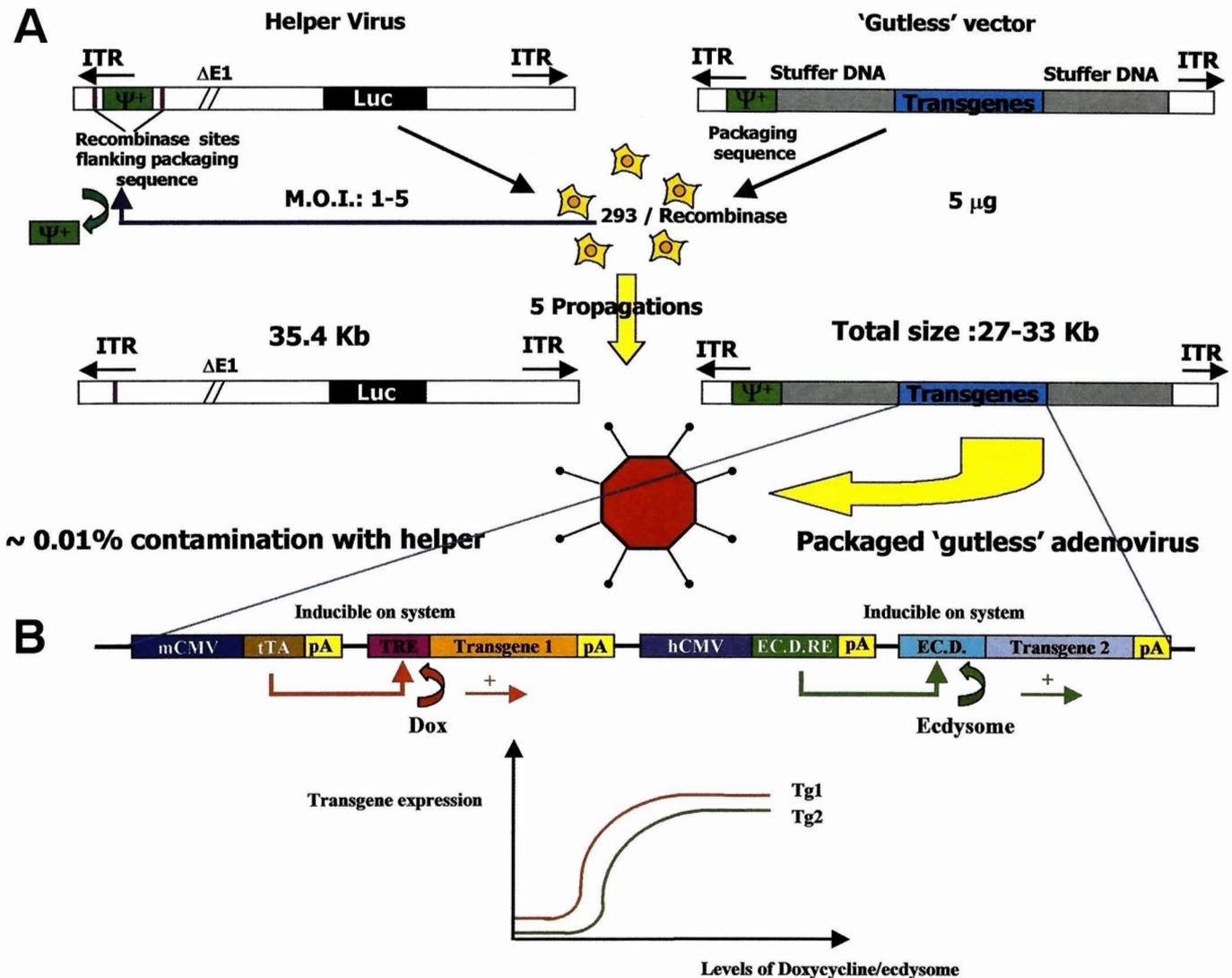


Fig. 2. A. Schematic description of the construction of a high capacity, helper dependent adenovirus. Producer cells, e.g. E1a expression 293 encoding a recombinase, are infected with a helper virus and transfected with a high capacity vector. The helper virus has been engineered to contain a packaging signal (Ψ) flanked by two sequences recognised by the recombinase. During replication the genomes of both the helper virus and the vector will be replicated. The recombinase will delete the packaging signal from the genome of the helper virus, leaving mainly vector genomes to be packaged into virions. **B.** Idealized vector genome of a helper dependent adenovirus. This figure shows a complex construct that would allow the expression of at least two different transgenes (Tg). Transgene Tg1, would be under inducible doxycycline-dependent transcriptional activator system (rtTA), while Tg2 would be under the control of the inducible ecdysone-dependent transcriptional activator (ECD). Currently, such large constructs cannot be inserted into any of the currently available vectors. As indicated below the scheme of the vector genome Tg1 would be expressed only in the presence of doxycycline and Tg2 would only be expressed in the presence of ecdysone. Thus, ideally, such a complete construct would allow to achieve a complex array of transgene expression and their fine regulation from a single viral vector.

Conclusions. Which are the future growth areas?

In summary, the main challenges to neurological gene therapy are the inflammatory and immunogenic potential of the vectors currently available, the complexities in delivering genes to normal brain cells and achieving very long term and widespread distribution of transgene expression with no adverse effects. All cells targeted by gene therapy are located beyond the cranium and the blood brain barrier, and most normal brain cells are postmitotic; this all poses limitations on our capacity to transfer genes into the brain. Major advances during the last five years have been: (i) the demonstration of the efficiency of viral vector mediated gene therapy in several animal models (Kostic et al., 1997; Choi Lundberg et al., 1998; Kordower et al., 2000), (ii) the establishment of viral vectors derived from retroviruses and adenoviruses, i.e. human lentiviruses and 'gutless' adenoviruses, which can infect and express transgenes in postmitotic cells such as adult neurons (Naldini et al., 1996b; Thomas et al., 2000), and (iii) the discovery of neuronal brain stem cells in the adult human brain (Erikson et al., 1998).

Another limitation to the use of viral vectors, are the inflammatory and immune responses they generate. Thus, various modifiers of the inflammatory and immune responses in the brain and periphery have been used, their clinical implementation has been rather limited. Again, it is here where human lentiviruses and 'gutless' adenoviruses provide an advantage: the potential for long term expression in the presence of reduced immune responses. Recently, the murine cytomegalovirus promoter has been engineered into recombinant adenoviruses to drive the expression of the marker gene, β -galactosidase. When delivered to the central nervous system (CNS), 1000 times less virus is needed to still have adequate levels of transgene expression, in complete absence of adverse immune reactions in the central nervous system (Gerdes et al., 2000). This poses a major advance towards clinical implementation of these strategies for chronic neurodegenerative disorders, since it will be possible to sustain high levels of therapeutic transgene expression in the absence of adverse neurotoxicity caused by inflammation.

Also, the possibility of predetermining the cell-type (i.e. neurones or glial cells) and the duration of therapeutic transgene expression in the CNS, constitutes a powerful tool for the implementation of gene therapy strategies for the treatment of PD (Smith-Arica et al., 2000; Castro et al., 2001). A vector which could be developed for the clinical implementation of gene therapy for PD, is shown in figure 2. It comprises of a gutless adenovirus backbone, harbouring two therapeutic transgenes under the control of two different inducible systems, i.e. the tetracycline and the ecdysome systems. The two therapeutic transgenes would be regulated independently from one another by the presence or absence of the inducers, i.e., doxycycline or ecdysome.

Also, when not needed, transgene expression could be switched off.

Physical barriers to the entry of vectors into the brain remain firmly unchallenged: although blood brain barrier disruption is used clinically to increase the entry of chemotherapeutic agents into the brain, its application to viral mediated gene delivery has not provided clear success. Equally, the problem of the diffusion of viruses, and proteins within the brain remains a limitation. Data from the laboratory of B. Davidson indicate that lysosomal proteins, which are normally secreted into the extracellular space may diffuse well within the brain (Ghosdsi et al., 1998). Interestingly, lysosomal proteins can be taken up by neurones, through the mannose-6-phosphate receptor. The engineering of proteins to be taken up by similar mechanisms may be a way to allow proteins to be taken up by neurones. Although many potentially interesting strategies exist and are shown to work reasonably well in experimental models, their success in gene therapy still depends on improvements in the capacity to deliver the actual genes to the brain using gene therapy tools. Also, more research is necessary to unravel the mechanisms and causes that lead to dopaminergic neurodegeneration in PD. A better understanding of this process would enable to encode other therapeutic transgenes that could be used to develop gene therapy strategies for PD.

Human neuronal stem cells constitute the other major potential advance towards the development of novel therapeutic interventions for the treatment of PD. The formal demonstration of their existence will now allow their cloning, characterisation, and eventual engineering using gene therapy strategies, for use in human patients. How these cells will work and integrate into the neuronal circuitry of the adult human brain constitute a challenging development.

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