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Gene Therapy for Parkinson's Disease: Towards Non Invasive Approaches

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1. Introduction

The blood brain barrier (BBB) is one of the factors hampering the development of new therapies and in many cases limits the access of the therapeutic agent to the neural tissue. The BBB consists of a specialized vascular structure formed by the interaction between endothelial cells and numerous processes of astrocytes regulating the passage and diffusion of molecules between plasma and the central nervous system (CNS) (Pardridge, 2005). The endothelium is distinguished from other tissues by the presence of tight junctions between endothelial cells (which limit the exchange of even low molecular weight substances) and a reduced endocytic and pinocytic activity. Small molecules (usually less than 500 Da) and some lipid-soluble small peptides can pass the BBB without the mediation of specific transporters. However, in most cases, the transfer across the BBB requires receptor-mediated transcytosis or selective transporters, such as low density lipoprotein receptors (LDLR), insulin receptors, leptin receptors, transferrin receptors and insulin-like growth factor receptors (Pardridge, 2005). The number of specific transporters mediating the transfer of substances through the BBB is very high and probably accounts for a very substantial part of the more than 2,000 membrane transporters that probably function in the human cell. We present, in this review, some of the newly developed approaches to overcome this barrier and facilitate the access of therapeutic genes to the nervous system.

Parkinson's disease (PD) is considered here as being neurological diseases for which no etiopathogenic treatment is available and may be susceptible to gene therapy. Different proteins such as human glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF)...which are able to reduce the vulnerability of dopaminergic neurons in laboratory animals have been described in the last ten years. For various reasons, the central administration of these proteins has not been effective in patients (short half-life, poor diffusion in neural tissue ...). Since the cellular and neurochemical substrate of the disease, and various proteins with neuroprotective capacity for these cells are known, gene therapy is outlined here as a future therapeutic option.

2. Gene therapy: An alternative treatment for neurodegenerative diseases

Conventional drugs of low molecular weight are designed to spread in cells with precise kinetics and, where necessary, use specific transport systems. Protein therapy is more complex, especially when act intracellularly, because there are not many cellular pathways

to import proteins. Moreover, these molecules cannot be orally administered. The situation regarding the delivery of nucleic acids is complex because of their size (MDa) and the lack of systems to import them through the cell membrane, especially in the cell nucleus. Therefore, nucleic acids need to be packaged into either virus particles in a natural way to meet many of these conditions or artificial particles that can replace the virus. The treatment half life is also completely different since the transformation which is achieved with nucleic acids can mean a permanent alteration, unlike conventional drug treatment which is inherently transient.

Gene therapy is defined as the introduction of nucleic acids into cells to alter the course of a medical condition or disease. Initially proposed for the treatment of monogenic diseases, gene therapy is now recognized as ' a new form of drug delivery ' offering various strategies for the treatment of inborn and acquired diseases. If the future of gene therapy is to successfully compete with traditional drug treatment, it will be necessary to have economic, simple and effective methods of gene transfer.

The expression of exogenously administered genes in the brain has been proposed as an alternative therapy for a wide variety of inherited and acquired diseases of the CNS for which classical drug therapy is not affordable or not readily applicable. Gene transfer into the CNS has been investigated as a strategy to protect against neuronal damage and degeneration. The best candidates for gene therapy are neurotrophic factors, antioxidants- or antiapoptotic molecules, and different specific molecules of cell signaling, which are of great interest in neuroprotection in pathological conditions where, as in Parkinson's disease, there is free-radical production (Tenenbaum et al., 2002).

The choice of vehicle (vector) to carry the therapeutic gene (transgene) to the desired tissue or cell type is crucial to achieve successful gene transfer. Basically, the vectors used in gene therapy can be divided into two groups, viral and nonviral, each of which has advantages and disadvantages. Some viral vectors which have proven to be effective *in vivo* gene transfer into the CNS (especially lentivirus and adeno-associated virus) can be integrated in the chromosomes of transduced cells, favoring a lasting expression of transgene in experimental animals (Thomas et al., 2003). In several cases, some cell lines are genetically transformed to produce neurotransmitters or neurotrophic factors in large quantities, called *ex vivo* gene therapy, in order to be used as therapeutic alternatives for CNS disorders. However, viral vectors present important problems regarding their production and safety (Kaiser, 2002). In addition, some viral vectors induce an immune response which reduces the effectiveness and biosafety with repeated dosing. A further complication of the use of certain viral vectors is their tendency to integrate near promoters and transcriptional units, thereby increasing the possibility of adverse effects (Essner et al., 2005).

A significant effort has been made to develop non-viral alternative strategies of *in vivo* gene transfer in recent years. Naked DNA linked to a variety of molecular conjugates, such as liposomes, non-lipid nanoparticles, polymers and polypeptides has been used to this end. The manufacture of large-scale DNA is feasible and reproducible with these vectors, and the final product does not require sophisticated storage conditions. In addition, nonviral vectors have no restrictions regarding the size of the gene and do not cause a significant immune response (Conwell & Huang, 2005). However, with the use of a non-viral vector, the input of genetic material into the cell is limited due to the need to provide the DNA in the cell surface in sufficient concentrations for entry (Luo & Saltzman, 2000). It is also difficult for non-viral vectors to induce a lasting expression of therapeutic gene (Conwell & Huang, 2005; Pathak et al., 2009). Although this limits the time for use in gene therapy of brain

diseases, it is also true that there has been spectacular growth, in recent years, in terms of diversity, properties and manufacturing.

The choice of an appropriate vector to transfer the desired gene in the affected brain area is crucial to establish a safe and efficient gene therapy for CNS. In recent years there has been much research into gene therapy, with significant progress in developing new gene transfer strategies in the CNS and in evaluating their potential in treating neurological diseases. Among the various systems developed for this purpose, viral vectors have undoubtedly been used most. However, due to the impediment of gene medicines in crossing the BBB, most of the work undertaken used adeno-associated viral vectors or lentiviral vectors (using invasive routes of administration such as intracerebral injection and craniotomy) that also produce a localized gene expression.

Although direct intracerebral injection of viral vectors expressing transgene-invasive gene therapy (iGT)-may be a reasonable alternative for localized treatment of neurodegenerative diseases, in which discrete brain structures are involved, the treatment of many neurological disorders requires the transfer of the transgene throughout the CNS. On the other hand, the small size of mice (major animal model) favors a more distributed gene expression, as only five vector injections are necessary throughout the brain. However, the larger human brain would require too many local injections, making the procedure clinically impractical. Therefore, in recent years there has been a dramatic shift in the strategies of non-invasive transfer of therapeutic genes in the CNS, non-invasive gene therapy (niGT) -. In this type of gene transfer, nucleic acids are indirectly introduced (usually via blood) in nervous tissue, in order to achieve therapeutic benefit, thus avoiding their direct injection into the brain parenchyma and damage to the BBB. The types and more important characteristics of non-viral and viral vectors that enable the realization of a niGT in the CNS will be described below.

3. Adenoassociated vectors: The most widely used in gene therapy for Parkinson's disease

The adeno-associated viruses (AAV) are small non-enveloped virus of 20-24 nm in diameter, which are not associated with any disease in humans (Blacklow et al., 1968). The 4.7-kb genome of the virus is packaged as a molecule of single- stranded DNA, it becomes double-stranded after infection. Recombinant AAV vectors (AAVr) lack 96% of the viral genome, which has been removed to leave only the two inverted terminal repeats involved in packaging and integration (Srivastava et al., 1983). The conventional method for generating viral stocks is to perform a transient cotransfection of 293T cells with plasmids containing the vector genome, rep and cap genes and adenovirus helper (Xiao et al., 1998). There have been recent improvements in vector production AAVr, resulting in higher titers (between about 100 and 10,000 times), and a higher proportion of infectious particles as well as free preparations of adenovirus (Zolotukhin, 2005). AAV vectors express stably transgenic products in dividing- and in non-dividing cells, and although the absence of viral genes theoretically minimizes the risk of activating the host immune responses, this will depend largely on the serotype used and the target tissue (Lowenstein et al., 2007; Wang et al., 2010). A small cloning capacity (3-4 kb) and a delayed expression of the transgene (2-3 weeks) can be included among the limitations of AAV vectors (Paterna and Büeler, 2002).

The human AAV was discovered in 1965 as a contaminant of adenovirus preparations. Following the description in 1982 of the first infectious clones of AAV2, this virus has been

rapidly gaining popularity in gene therapy applications due to features such as the lack of pathogenicity, wide range of infectivity, and the ability to set a long-term transgene expression. As more serotypes are characterized and more capsids of different serotypes are combined to obtain new tropisms, it is possible that tissues which are difficult to infect with known serotypes of AAV are capable of gene transfer. This will help to expand the current range of AAV vectors. AAV serotypes share a common genomic structure but show variation in cell and tissue tropism due to differences in their capsid proteins. This involves recognition of different cell surface receptors.

So far we have identified nine serotypes of AAV vectors, and the studies with reporter genes have shown that these vectors efficiently infect the neurons (Cearley et al., 2008). The receptors responsible for the infection of AAV vectors in neurons are not fully characterized. The coinfusion of heparin and mannitol increases the diffusion of AAV2 vectors. In addition, the activity of coreceptors is necessary for a successful viral entry (Qing et al., 1999). Previous mannitol intravenous infusion in mice not only induces a global transduction in the CNS, facilitating the entry of AAV2 which is also injected intravenously, but also produces therapeutic benefits in models of neurological diseases in which there is a generalized affectation (McCarty et al., 2009). The cells which are permissive to AAV2 vectors express a number of factors, including the fibroblast growth factor receptor and the β5 subunit of integrin, αvβ5 (Qing et al., 1999; Summerford et al., 1999). Compared to the AAV2 vector, the AAV5 serotype produces a higher- transduction and distribution in the brain (Davidson et al., 2000). Thus, AAV transduction in any organ is closely linked to the local density of a specific receptor.

AAV infection is initiated by binding receptors that are serotype-specific to the cell surface. For example, heparin sulfate proteoglycan (HSPG) is one of the main AAV2 receptors (Summerford & Samulski, 1998). Binding to a receptor is not a sufficient stimulus for viral internalization, which also needs coreceptors, such as integrin heterodimers , fibroblast growth factor type 1 receptor and hepatocyte growth factor receptor, c-Met (Dos Santos & Beyer-Nardi, 2008). HSPG has a ubiquitous distribution in cells and tissues of various species. This partly explains the broad tropism of this virus into human, nonhuman primate, canine, murine and bird cells. Some derivatives of sialic acid act as AAV4 and AAV5 receptors, while the platelet-derived growth factor receptor is another cellular determinant involved in AAV5 infection (Di Pasquale et al., 2003). Different AAV serotypes preferentially transduce different cell types. In general, tropism is associated with the abundance of a specific receptor /coreceptor, although there are exceptions (Duan et al., 2000). This suggests that there are probably other internalization ways that are independent of known receptors (Ding et al., 2005).

In the past decade, viral gene transfer has progressed from being merely an application in animal research to become an experimental therapeutic strategy in humans. From the clinical and therapeutic standpoint it is essential to know the distribution in tissue and kinetics (onset, duration and removal of the expression) of AAV vectors. To date numerous AAV serotypes with variable tropism have been identified. The level of homology of amino acids in the capsid protein of serotypes 1 to 9 is about 45% (Chiorini et al., 1999; Gao et al., 2002), with the most divergent serotypes being AAV4 and AAV5. Many studies have conducted evaluations on the tropism of different AAV serotypes. Serotypes 1, 2 and 5 (Burger et al., 2004), 7-9, and rh10 (one serotype of primates), 1, 2, 5, 7 and 8 (Taymans et al., 2007), 2, 5, 8 and rh10 (Sondhi et al., 2007), 8, 9, and rh10 and rh43 (Klein et al., 2008) have been used in the brain.

An important limitation of previous studies was that the lack of uniformity in the design of plasmids, as in the production, purification and route of administration, made it difficult to compare the efficiencies of transduction of these serotypes in different tissues. In order to control the variability in these parameters, the design of plasmids, transfection, purification, titration, dialysis, storage and injection have been standardized for all AAV serotypes examined. These limitations are solved by the use of marker genes in which expression can be viewed at different times, via longitudinal studies in each animal. Thus, when using luciferase as a transgene one can evaluate the transgenic protein activity after intraperitoneal injection of its substrate (luciferin). This allows sequential measurements in the same animal (Lipshutz et al., 2001; Wu et al., 2001). Consequently, the differences in the efficiencies of transduction and biodistribution profiles will only reflect the differences in capsids of the virions of these serotypes.

The tropism and expression kinetics for 9 different serotypes of AAV have recently been studied, which have been packaged, produced, purified and injected by the same systemic route (Zincarelli et al., 2008). This administration route is therapeutically realistic, as it allows the specific expression in tissues, provided that specific promoters are used along with the administration of vectors. In the afore mentioned study a sequential follow up of transgene expression for 100 days, and 9 months after the intravenous administration of serotypes 1-9 of AAV that expressed the luciferase transgen (controlled by an early promoter/activator of CMV and flanked by ITR sequences of AAV) was carried out in mice. The bioluminescence image revealed three expression levels: a) serotypes AAV2, 3, 4 and 5 were in the low expression group, b) AAV1, 6 and 8 belonged to the moderate expression group, and c) and AAV7 and 9 corresponded to the high expression group. There was great variation in the kinetics of expression between these serotypes, where AAV7 and 9 induced a faster expression onset, and AAV3 and 4 induced a slower expression. It has been also shown that the primary targets for serotype 1, 2, 5, 6, 7 and 9 are liver and skeletal muscle, and was AAV9 whom induced greater gene expression in the heart. AAV8 and 9 induced a more ubiquitous transduction than other serotypes, but AAV9 generated a more robust expression. Within the high expression group, AAV9 also produced the largest number of viral genome copies in all examined tissues (especially liver, heart and skeletal muscle) except in the testes and lung. Significantly, the expression in the testes is an important safety issue for future clinical research with this serotype.

AAV particles are currently the preferred vehicle for gene transfer in the CNS, because they are not pathogenic, they can transduce postmitotic cells (neurons, astrocytes and oligodendrocytes) (Carter & Samulski, 2000; Monahan & Samulski, 2000), persist in cells without causing insertional mutagenesis, allow a long-term gene expression in the brain (important for the treatment of chronic disease) and spinal cord (Lo et al., 1999; Wu et al., 1998) as well as peripheral nervous system, with no associated immune response (Chen et al., 2007; Federici & Boulis, 2007). In addition, AAV vectors are safe, non toxic and incompetent for replication. The use of AAV vectors for trophic factor gene transfer to CNS also provides the added benefit of producing physiological levels of the protein, thereby avoiding side effects associated with large doses (Tenenbaum et al., 2000). Since the first successful application in 1984 using serotype 2 of AAVr (AAV2) as a vehicle for gene transfer, numerous AAVr serotypes have been isolated and characterized. Although many of these viruses have good transduction efficiencies in various body organs via simple administration, generalized transduction of CNS with relatively non-invasive methods is ineffective. We have commented previously that the BBB acts as a protective barrier that

excludes potentially harmful molecules and organisms according to their size, charge and lipid solubility. Consequently, the BBB effectively blocks the spread of AAVr in the CNS (Manfredsson et al., 2009). Researchers then had to resort to directly injecting viral vectors into the brain. These injections produce a robust transduction, but are relatively local and require invasive neurosurgery.

There are currently AAV vectors expressing different capsid proteins with the ability to transduce different cell types of the CNS. Transduction varies depending on the AAV serotype used and the injected brain region. In the mouse brain, AAV1 and 5 can transduce neuronal cells and glial cells, while AAV2 can only transduce neurons (Davidson et al., 2000; Wang et al., 2003). AAV2 can only transduce cells of the nervous system located in the vicinity of the injection site. This implies the need for multiple injections or using agents such as mannitol or heparin to cover larger areas of the CNS (McCarty et al., 2004; Nguyen et al., 2001). After having examined the characteristics of transduction in the adult mouse brain, the following four new serotypes have recently been discovered in primates: AAV7, 8, 9 and Rh10 (Cearley & Wolfe, 2006). Some of the AAV serotypes previously characterized , such as AAV1, AAV2 and AAV5, have varying levels and specificity of transduction (depending on the site of injection) when administered directly into the brain. For example, a large proportion of dopaminergic neurons in the substantia nigra are infected with an AAV2 vector, and the transduction efficiency and diffusion is much lower in the striatum (Björklund et al., 2000).

Most information about efficiency and specificity of AAV vectors for treating CNS disorders comes from studies in animal models in which different vectors are evaluated according to their potential to ameliorate the symptoms of a disease or syndrome. The first clinical trial data in describing gene transfer mediated by AAV vectors in Parkinson's disease, Alzheimer's disease, and Canavan (an autosomal recessive degenerative disorder) are now being reported.

Although invasive strategies for gene therapy are used or proposed for neurodegenerative diseases there are now new serotypes of AAV (as AAV9) that enable non-invasive transfer of transgenes in the CNS.

4. Current gene therapy strategies for Parkinson’s disease

Three strategies of gene therapy in parkinsonian patients were first tested in 2006, two were aimed at achieving direct symptomatic benefit and the other was aimed at preventing the natural course of the disease. In the first study, the gene transfer of aromatic amino acid decarboxylase (AAD, an enzyme that metabolizes levodopa to dopamine) to the striatum of PD patients was carried out, using AAV2 (Eberling et al., 2008). The aim was to optimize the symptomatic response to levodopa administration which could then be metabolized to dopamine more stably and constantly. However, the most convincing results were achieved with a triple enzyme transfer approach delivery, using the GTP cyclohydrolase 1 gene (GCH1) in addition to tyrosine hydroxylase (TH) and AAD genes (Shen et al., 2000; Azzouz et al., 2002; Muramatsu et al., 2002).

Dietary tyrosine (Tyr) passes the blood–brain barrier and is taken up and processed by the dopaminergic neuron. Tyr is converted by TH into L-3, 4-dihydroxyphenylalanine (DOPA), which is in turn converted by the aromatic amino acid decarboxylase into dopamine. TH is the rate-limiting enzyme in the dopamine synthesis pathway and its activity is highly dependent on the presence of tetrahydrobiopterin (BH4) which acts as a cofactor. BH4 itself

is synthesized from GTP in a three-enzyme reaction where the activity of the GCH1 enzyme is rate limiting. A lentiviral vector of non-human origin was used in the triple enzyme transfer approach, now commercially known as Prosavin , which includes the three necessary genes for dopamine production and where the TH gene lacks the coding region for the regulatory N-terminal part of the enzyme to avoid negative feedback from cytosolic dopamine (Björklund et al., 2010). A clinical trial using this vector was started in 2007. One drawback of the two approaches that are now being used in clinical practice for enzyme replacement for treatment of PD patients is that the vectors used almost exclusively transduce the intrinsic striatal neurons, i.e., cells that do not normally produce dopamine (Björklund et al., 2010)). Therefore, it is conceivable that the accumulation of cytosolic dopamine and its end products will expose the transduced cells to excessive oxidative stress, since striatal transduced cells lack the vesicular storage and release mechanisms, possibly leading to toxic damage or neurodegeneration (Chen el al., 2008; Björklund et al. 2010). Moreover, non-regulated dopamine production induced by overexpression of aromatic amino acid decarboxylase may trigger or aggravate dyskinesias (Bankiewicz et al., 2006; Eberling et al., 2008).

The same AAV vector for gene transfer of glutamic acid decarboxylase to the subthalamic nucleus was used in the second approach. The aim was both to facilitate GABAergic transmission in this region, thereby reducing the overactivation of subthalamus-nigral glutamatergic neurons that mediate a substantial part of the characteristic PD disorders (in a manner analogous to that induced both by lesions and deep brain stimulation in that region) and to normalize output from the nucleus (by adding an inhibitory GABA outflow, thereby reducing excessive excitatory glutamate output to key targets such as the globus pallidus interna and the substantia nigra reticulata). This strategy has also reached phase I clinical trials: the unilateral injection of AAV2-GAD into subthalamic nucleus showed that this procedure was safe and was associated with improvements of parkinsonism (Kaplitt et al., 2007). Although studies of other gene, cell and biological therapies in patients with PD have also shown promise in small, open-label studies, subsequent randomised double-blind clinical trials have not substantiated their initial findings (Marks et al., 2010). A double-blind randomised trial was recently conducted at seven centres in the USA specialising in PD care and functional neurosurgery (Lewitt et al., 2011). This trial was conducted to assess the effect of bilateral delivery of AAV2-GAD into the subthalamic nucleus compared with bilateral sham surgery in patients with advanced PD. The use of this approach in this small phase 2 trial was intended to serve mainly as a proof-of-concept study, and the authors found evidence of a benefit of subthalamic nucleus AAV2-GAD surgery versus sham surgery. The authors also commented that several secondary outcome measures did not show improvement in the AAV2-GAD treatment group compared with the sham group. This result might indicate the absence of a benefit or relate to the fact that this small phase 2 trial was likely to be underpowered for some of the clinical outcomes that are more difficult to achieve, such as, improvement in function, daily living activities and quality of life measures (Lewitt et al., 2011). Subsequent larger clinical trials will be needed to assess whether this treatment is practical for more widespread clinical use.

The third approach uses CERE-120, an AAV2-based gene transfer vector designed to deliver the neurotrophic factor human neurturin (NRTN) to these degenerating neurons as a treatment for PD. Neurotrophic factors are naturally-ocurring proteins that restore function and prevent death of damaged neurons. A phase 1 trial in 12 moderately advanced PD patients did not identify any safety issues, while suggesting possible improvements in

several measures of motor function (Marks et al., 2008). A subsequent double-blind-controlled Phase 2 trial in 58 subjects further supported the safety of CERE-120, but failed to discern any benefit compared to sham surgery on the primary endpoint (UPDRS-motor-"off" at 12 months) although there were hopeful signals in the subgroup with longer follow-up and in secondary measures (Marks et al., 2010). Two PD patients in the Phase 2 trial died from non-AAV2-NRTN-related events, and their brains were examined histologically. Although results showed NRTN-immunostaining in the targeted striatum, however, only scarce evidence of neurturin was seen in the substantia nigra compacta (SNc) cell bodies in PD (Bartus et al., 2011). These authors have proposed that human PD is associated with an underlying defect in axonal transport such that there is no effective delivery of NRTN from the source in the putamen to the dopaminergic neurons in the SNc. Their observations also suggest that the transport defect in PD is widespread in the brain: they not only noted deficient retrograde transport to the SNc, but also an absence of anterograde transport to the SN pars reticulata, suggesting that the disease produces defects in transport in dopaminergic and non-dopaminergic neurons (Bartus et al., 2011; Lewis and Standaert, 2011). In animals, Cere-120 not only increases the endogenous synthesis of dopamine, but also protects dopaminergic neurons from the effects of various neurotoxic agents (Kordower et al., 2006), rescuing them from the degenerative process (Herzog et al., 2007). As is the case in any experimental therapy, caution is paramount, but balanced against the potential for a transformative therapeutic breakthrough, it seems right to proceed with further clinical studies.

5. AAV9: The viral vector crossing blood brain barrier

The ability of AAV type 9 (AAV9) to cross the BBB after intravenous infusion (both newborn and adult mice) and transduce large areas of the brain and spinal cord has recently been reported (Foust et al., 2009). This finding has been subsequently corroborated by a second independent group (Duque et al., 2009). The results obtained are of great importance, given the longstanding interest in developing vectors that could cross the BBB. A single intravenous AAV9-GFP injection to newborn mice induced a wide transduction in neurons throughout the CNS; For example, transduction was observed in almost 60% of motor neurons of the spinal cord, in about 65% of Purkinje cells, and 10-20% of neurons in the cortex and hippocampus. Differential exposure of a receptor on the cell surface may explain the propensity of the virus to transduce a larger diversity of neurons in the brain after i.v. injection. However, intraparenchymal injection of the vector affected neurons located in regions that were poorly transduced after systemic injection, for example, the striatum. These results have raised some questions (Saunders et al., 2009): what the role of hemodynamics in the circulation of the virus is and if transduced neurons are simply a product of a greater blood flow reaching them.

Transduced cell types changed from neuronal to glial when AAV9-GFP was intravenously injected in adult animals. Thus, transduction of astrocytes and to a lesser extent of microglial cells was observed throughout the CNS, with nearly 60% of astrocytes in the spinal cord being transduced. It is interesting to note that the AAV9 mediated glial transduction was seen only after intravenous infusion, whereas intraparenchymal injection induced the classical neuronal pattern of transduction. Why does glial transduction depend on the route of administration? It is possible that viral receptors are only expressed in feet of astrocytes that line the brain blood vessels, therefore, restricting access to neurons (Abbott, 2005).

The AAV9 entry mechanism into cells is still to be resolved, as understanding the polarity of the astrocytic transduction can help determine AAV9 receptors. However, it was recently reported that terminal N-linked galactose is the primary receptor for AAV9 (Shen et al. 2011). In the study by Foust et al (Foust, et al., 2009), it appears that AAV9 peripheral administration in adult mouse infected astrocytes, once the virus interacts with the feet of perivascular astrocytes which are in direct contact with vascular endothelial cells. These cells may contain a population of receptors different from those exposed when making direct injections into the brain parenchyma, and the AAV9 mediated transduction properties were especially interesting. Even if the BBB were opened in the adult by the use of drugs, one would not expect that the particles which were still AAV of 80 nm to cross the BBB, thereby preventing the free access to potential AAV receptors in brain parenchyma (Manfredsson et al., 2009). However, following intravascular delivery, AAV9 and other vectors crossing BBB also target liver, heart, skeletal muscle and other tissues, which may cause untoward effects. A major challenge is to confine transgene expression to the CNS when rAAV9 is delivered systemically. Historically, CNS specific promoters have been used

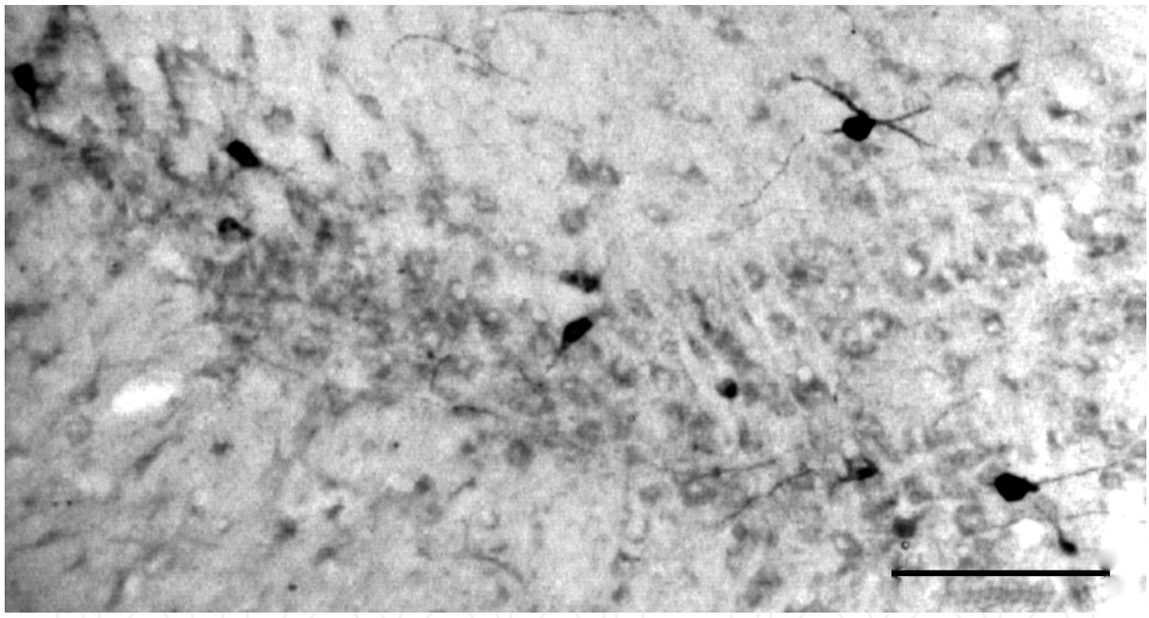

Fig.1. Intravenous injection of GFP-encoding AAV9 mediates substantia nigra transduction in old C57BL/6J mice (twenty months old). A substantia nigra anti-GFP immunohistochemistry staining was carried out after 6 weeks of AAV9 i.v. injection ($4x10^{11}$ vg in 200 µl). GFP^+ cells can be observed with different intensities of expression. Scale bar 500 µm.

to limit transgene expression to the CNS. However, tissue-specific, strong CNS promoters are often too large to be packaged into the rAAV genome. Several potential challenges remain to using rAAV9 to treat CNS disorders. The first challenge is to deliver rAAV specifically to the CNS. As an alternative, tissue-specific endogenous microRNAs (miRNAs) were recently used to repress rAAV expression outside the CNS, by engineering perfectly complementary miRNA-binding sites into the rAAV9 genome (Xie et al., 2011). MicroRNAs (miRNAs) are a unique class of short, non-coding RNAs that mediate post-transcriptional

regulation of gene expression ranging from developmental processes to disease induction or amelioration. The authors reported the use of miRNAs to detarget rAAV9 expression both separately and concurrently in the liver, heart and skeletal muscle which are the three tissues that are most efficiently targeted by intravenously delivered rAAV9 (Zincarelli et al., 2008; Xie et al., 2011).These are promising findings which could facilitate the development of miRNA-regulated rAAV for CNS-targeted gene delivery and other applications.
AAV9 employment may have important implications for the treatment of PD (Fig.1).
Neurotrophic factors that are secreted, such as GDNF and Neurturin, are important therapeutic agents which have been proposed for the treatment of PD. Since most of these factors are secreted by glia it is easy to imagine that overexpression of trophic factors AAV9 mediated in these cells should produce a significant improvement in cell rescue, similar to or better than that found when the same neurons are therapeutic targets.

6. Nanoparticles-based vectors crossing blood brain barrier

Almost 70% of current clinical trials use viral vectors as vehicles for delivering DNA into cells to repair defective genes. Although viral vectors are effective and are widely used, it is also true that there are still some important safety aspects to be considered when viral particles are used in a therapeutic program (Thomas et al., 2003). The real goal is to achieve an efficient, non-invasive and non-viral gene therapy for the brain because of the obvious side effects of the viral vectors. This will require multidisciplinary approaches from different fields such as engineering, chemistry, cell biology, physiology, pharmacology and medicine. Although this ideal scenario has not been reached yet, a considerable amount of work on nanotechnological gene-delivery strategies to cross the BBB has been done.
The last fifteen years have seen the beginning of another scientific revolution, other than gene therapy, based on the ability to measure, manipulate and organize matter on the nanometer scale. Nanotechnology is defined as the set of knowledge and methodologies to study, manufacture and characterize functional structures with dimensions of less than a few tens of nanometers. The application of nanotechnology in biomedical research is having a significant impact on the development of new types of diagnostic and therapeutic tools. A key focus is the development and use of non-viral vector-based nanoparticles to bring about safe and efficient gene transfer. Among the major advantages of nanoscale vehicles for transfer of drugs is their ability to cross membrane barriers, particularly in the CNS.
The term "nanoparticle" refers to a structure with a diameter in the range of 1 to 100 nm, and was among the first nanoscale materials to have been directly used in biological systems. They must be functionalized in some way to be effective, which means the ability to be filled with, or attached to, therapeutic molecules (such as drugs, nucleic acids ...) or labeled with antibodies or nucleic acids to facilitate detection of a target of interest. They can be made by way of nanocrystals, drug-polymer complexes, or by creating nanoscale spheres (liposomes) able to catch molecules of drugs or other agents (LaVan et al., 2003).
Polymeric nanoparticles have been effective in gene transfer studies (Cohen et al., 2000). They are colloidal particles carrying drugs or genes of interest within a biodegradable polymer matrix. Depending on the method of preparation, nanoparticles, nanospheres or nanocapsules may be obtained. Nanospheres consist of a polymer matrix in which the drug or gene are physically and uniformly dispersed, while nanocapsules represent vesicular transport systems in which the drug or gene is confined in a cavity surrounded by a matrix polymer. Polymeric nanoparticles are more efficient in terms of transport of drugs /genes

compared with traditional oral and intravenous methods (Soppimath et al., 2001). These advantages stem from two basic properties: 1) their small size favors penetration through small capillaries, allowing a greater accumulation of the drug /gene at the target site (Soppimath et al., 2001), this is particularly relevant in the CNS, in which the transport of some drugs is limited because of their inability to cross the BBB and 2) the application of nanoparticles as vehicles for transport of drugs / genes may help overcome this obstacle. In fact, it has recently been shown that polymeric nanoparticles are effective for the transport of peptides and other agents through the BBB (Kreuter et al., 2003; Nahar et al., 2006). The use of biodegradable polymers favors the sustained release of drugs / genes into the target site over a long period (Tang et al., 2009).

Dendrimers are highly branched three-dimensional macromolecules surrounding a central core, which can be designed at the nanoscale with remarkable precision. Dendrimers have a number of free ends, to which molecules of different nature, from therapeutic agents to fluorescent molecules can be attached and transported. Different drugs or DNA molecules can be incorporated at the core, and because of its branched structure, a single dendrimer can carry a high number of molecules compared to other transport systems based on nanoparticles. Multiple terminal groups which are predominantly located on the surface may control the interaction of the macromolecules of dendrimer with their molecular environment. In fact, dendrimers tend to contain more than 100 end-groups, with diverse reactive sites to allow conjugation with different types of molecules (Jain & Gupta, 2008). Moreover, these end groups can be modified to make their interior hydrophilic and to keep their exterior hydrophobic, or vice versa (Sahoo &Labhasetwar, 2003). It has recently been shown that dendrimers can be promising gene transfer vectors for Parkinson's disease. Examples will be included later.

Fullerenes are small spheres of a few nanometers in size (nanospheres), consisting of carbon atoms located in such a way as to form nanoscale hexagonal and pentagonal structures. The best known fullerene is the carbon 60 (C-60), which consists of 60 carbon atoms forming a structure similar to that of a soccer ball. A water-soluble fullerene, derived from the C-60, capable of crossing the cell membrane and that it mainly located in mitochondria has been reported (Foley et al., 2002). This opens up great possibilities for mitochondrial gene therapy, for example, in Parkinson's disease, bearing in mind the important role of mitochondria in this disorder. The carbon nanospheres derived from glucose are an emerging class of intracellular vectors. The surfaces of these spheres are highly functionalized and require no further modification. The *in vivo* experiments have shown that these nanospheres can cross the BBB and are identified in the brain as well as in the liver and spleen (Selvi et al., 2008; Wong-Ekkabut et al., 2008). There is also evidence that nanospheres are continually being removed from these tissues over time.

Although non-viral vectors based on nanoparticles are easily produced and have low immunogenicity, there are issues about toxicity, specificity, regulation of transgene expression and transfection efficiency which need to be resolved before clinical application.

7. Application of nanoparticles in gene therapy for Parkinson's disease

The preferred strategy, in gene therapy of diseases affecting large areas of the brain, would be to administer vectors systemically. The human brain contains around 100 million capillaries covering a surface of approximately 12 m^2 (Bickel et al., 2001). Virtually every neuron in the brain has its own capillary, with an average distance of capillary to neuron of

8-20 microns (Schlageter et al., 1999). The administration of a therapeutic gene to neurons through the capillary membrane would then be the method of choice. However, we have previously seen that the BBB is a serious obstacle to the entry of macromolecules in the brain.

The basic mechanism, coined by Pardridge as molecular Trojan horses, is that a protein or DNA that is going to cross the BBB is coupled /conjugated to a ligand that is recognized by a receptor which is present on the luminal side of capillary endothelial cells of the brain. Once in the blood, the complex protein / DNA-ligand bind to the receptor, performing a process of endocytosis. This complex then moves through the endothelial cytoplasm, thereby avoiding the endosomal / lysosomal system, where it is then left on the abluminal side (brain). This system of transporting proteins across the BBB has been successfully used for vasoactive intestinal peptide, BDNF, epidermal growth factor, but also as pegylated immunoliposomes (ILP) containing plasmid DNA expressing β-galactosidase, tyrosine hydroxylase and GDNF , among others (Zhang &Pardridge, 2009).

We have previously mentioned that the BBB has specific receptor-mediated transport mechanisms, which can be used as a transport pathway of drugs/genes to the brain. The transferrin receptor is particularly interesting because its expression is restricted to the brain capillaries and to neuronal membranes (Jefferies et al, 1984). ILP have been used to deliver genes for the targeting of the brain with colloidal carriers. The transfer of pegylated immunoliposomes from the blood to the brain is achieved by monoclonal antibodies targeted against the transferrin receptor or insulin, which, by binding to their respective ligands induce receptor-mediated endocytosis (transcytosis), then subsequently incorporate foreign genes into the brain parenchyma without damaging the BBB. The gene for tyrosine hydroxylase (TH) has been expressed with intravenous administration of pegylated immunoliposomes in a PD model with 6-hydroxydopamine, which revealed the normalization of expression levels of TH in the striatum (Zhang et al., 2003).

Compared with liposomes, pegylated nanoparticles are physically and chemically more stable and can be lyophilized for long term storage. The pegylated nanoparticles conjugated with cationic albumin (CBSA-NP) may also be used for the transfer of genes to the brain (Lu et al., 2005). Therefore, more CBSA-NP marked with a fluorescent probe are accumulated in mouse brain cells (after being injected intravenously) than when using pegylated nanoparticles conjugated with native albumin. CBSA-NP incorporating plasmid expressing cytotoxic genes have been used for non-invasive experimental gene therapy of gliomas (Lu et al., 2006). Thus, for the first time, the transfer of a cytotoxic gene using a noninvasive route in the mouse brain to treat a malignant glioma has been demonstrated. The current findings encourage further studies into the application of CBSA-NP for noninvasive gene therapy of PD.

Polyamidoamine dendrimers (PAMAM) have recently emerged as a new class of nanoscale spherical polymers to have caught the eye of researchers from various scientific disciplines. It is increasingly clear that PAMAM is a multifunctional polymer with many applications, such as transfer vehicles for antisense oligonucleotides and siRNA (Kang et al., 2005). Moreover, in itself, PAMAM can behave as an efficient gene carrier. PAMAM possessing primary amino surface groups have the inherent ability to associate with and condense DNA, and have been used efficiently in biocompatible DNA transfer (Kim et al., 2004). Good efficiency of transfection is also achieved by modifying the surface of PAMAM with L-arginine. The primary amines located on the surface of these dendrimers allow conjugation with some ligands such as transferrin, to achieve efficient gene transfer to the brain. A

vector for gene transfer in the brain was recently developed with this in mind. Remember that the transferrin receptor is expressed in the BBB and the neuronal membrane (Jefferies et al, 1984). Intravenous injection in mice of a nanoscale highly branched dendrimer modified with transferrin and PEG (a hydrophilic polymer that increases the biocompatibility of the vector) (PAMAM-PEG-Tf), induces a higher brain expression (almost double compared to other dendrimeric vectors) of an exogenous gene encapsulated in the vector (Huang et al., 2007a).

Intravenous administration of PAMAM dendrimers conjugated to lactoferrin (Lf) using bifunctional PEG allows the efficient expression of exogenous genes in the brain. As a ligand targeting the brain, the Lf can bind specifically with Lf receptors of brain cells. The Lf is an iron-binding glycoprotein belonging to the transferrin family. One of the advantages of Lf as a ligand for gene therapy in brain is the low plasma concentration of endogenous Lf (5 nM). Its plasma concentration is much lower than the kd of Lf receptors in the BBB (Huang et al., 2007b). This efficiently prevents the competitive inhibition of endogenous Lf with exogenously transference systems of genes/drugs cojugated to Lf. In addition, Lf transport across the BBB monolayer model appears to be unidirectional i.e. from apical to basolateral side. This unidirectional transport could result in a greater neuronal accumulation of such systems of Lf conjugates when compared to conjugates of transferrin. These advantages are partially reflected in a recent study showing that exogenous gene expression in the brain, mediated by nanoparticles conjugated to Lf is almost 5.2 times that obtained with unconjugated nanoparticles (Huang et al., 2008). *In vivo* image analysis has shown that Lf-nanoparticles accumulation was higher in the brain (but lower in other organs) than their unmodified counterparts (Huang et al., 2010a). A recent study has reported significant neuroprotective effects with Lf-nanoparticles carrying the GDNF gene, after being administered intravenously in a rat model of PD induced by the pesticide substance rotenone (Huang et al., 2010b).

However, the application of different transfer protocols has been limited by the lifetime of the protein in circulation, the need for repeated injections or low transference yields achieved in the brain. The recently published paper by Spencer and Verma gives a possible solution to the first two issues (Spencer & Verma, 2007).

The low-density lipoprotein (LDL) receptor (LDLR) family consists of a cell-surface receptors group that binds lipoprotein complexes for internalization to the lysosomes. This family consists of about 10 different receptors that are expressed in a tissue-specific manner and primarily bind apolipoprotein complexes (Brown & Goldstein, 1986). The apolipoproteins, whose more representative members are apolipoprotein B and apolipoprotein E, are attached to blood lipids in order to direct them to lysosomal degradation. Apolipoproteins bind to the LDLR on the cell surface of the targeted cell, and thereby the complex is endocytosed. In contrast, when LDLR binds to apolipoproteins in BBB it induces transcytosis to the abluminal side of the BBB and, presumably, the apolipoprotein is released and taken up by neurons and/or astrocytes (Hussain et al., 1999). Spencer and Verma used a lentivirus vector system to deliver the lysosomal enzyme glucocerebrosidase and a secreted form of GFP to the neurons and astrocytes in the CNS. These authors fused the LDLR-binding domain of the apolipoprotein B to the targeted protein, moreover adding a secretory leader sequence to allow its release. It took a single intraperitoneal injection of lentiviral vector for the protein could be detected two weeks later in the CNS, showing that it had entered through transcytosis by binding to the LDLR (Spencer & Verma, 2007).

The interesting thing about this therapeutic approach is that the lentiviral vector can also deliver genes to a peripheral organ (liver or spleen), which now serve as a source for the prolonged and continuous expression, and secretion of a therapeutic protein which has the ability to cross the BBB. This technique could be used to facilitate the passage of neuroprotective molecules, of interest in Parkinson's disease, through BBB. But there are still some unresolved issues, for example, the immunogenicity of the fusion protein and the restricted cerebral distribution of LDLR, as only a minority of brain cells are transfected. The first problem will be difficult to address while the latter may possibly be solved using other receptors that activate transcytosis, such as the diphtheria toxin receptor (De Boer & Gaillard, 2007).

8. Towards a non invasive gene therapy for Parkinson's Disease

Direct administration of transgenes in neural tissue (iGT) has advantages for the treatment of certain neurological diseases because it avoids the peripheral expression of the transgene (and related immune activation), and widespread central expression (unnecessary in focal neurological diseases). However, iGT also has significant drawbacks, most notably the use of surgical procedures (with BBB damage, nonspecific reactive gliosis, risk of infection ...) that cannot be repeated many times in the case of chronic diseases. In addition, this mode of administration usually induces intense effects on the injected brain regions, but does not diffuse well in the surrounding tissue (exponential decrease of transgene expression with distance from the injection point), and making it difficult to adjust the therapeutic dose. In contrast, non-invasive gene therapy (niGT) allows greater distribution of the transgene in the brain (particularly useful in diseases with diffuse brain affectation) and does not require surgery, thus allowing repeated administration in chronic neurological diseases (the most enduring expression of the transgene has been reported for AAV and has always been less than 10-15 months).

All gene therapy clinical trials for the treatment of PD have used the iGT, which makes sense when the therapeutic objective is aimed at preventing the degeneration of dopaminergic neurons or treating the primary symptoms associated with a specific motor area. However, it is becoming increasingly clear that from its initial stage, PD does affect non dopaminergic neuronal populations of different brain centres (brain cortex, olfactory bulb, *pedunculopontine nucleus*, *locus coeruleus* ...), inducing symptoms that go far beyond the original motor disorders associated with dopaminergic transmission (autonomic disorders, dementia, attentional disorders, sleep disorders, depression ...). For example, the anatomical substrate of depression has been associated with changes in either noradrenaline, serotonin and dopamine transmission in various centers such as the locus coeruleus, raphe nuclei, or various centers of the basal ganglia and the limbic system (Levy & Dubois, 2006; Remy et al., 2005). While in PD without dementia, cognitive impairments are associated with abnormalities in dopamine transmission in the cerebral cortex and ventral striatum (Owen et al., 1995), PD with dementia also includes disorders in cortical cholinergic transmission (Klein et al., 2010). Therefore, future gene therapy for this disease should target not only the dopamine centres associated with motor disorders, but also other centres and other neurotransmitters. From this perspective, the greatest extension of the effects of niGT (compared with iGT) might be particularly useful in patients with widespread central affectation; such therapeutic actions would be difficult to achieve using iGT.

9. Towards a regulatable gene therapy for Parkinson's Disease

All current gene therapy approaches for Parkinson's disease use non-adjustable constituent vectors, so that in all cases the transgene expression, once introduced in the body, escapes any external-non-adjustable gene therapy (nrGT). It is often true that animal studies show that therapeutic responses induced by nrGT are achieved with significantly lower doses than those capable of inducing undesirable side effects. In fact, in the three annotated gene therapies for PD, the dose which is intended to produce therapeutic responses is significantly lower than that for which undesirable side effects would be expected, suggesting an acceptable safety profile even when using non adjustable constituent vectors (although the available evidence in humans comes from only relatively small samples of patients who have only been followed for short periods after treatment).

Bearing in mind these considerations and taking into account the extensive AAV2 information evidencing either its low toxicity, lack of regulated promoters shown to be safe and effective in patients, and urgent clinical need in numerous neurological diseases (particularly PD), some authors have suggested that the use of adjustable vectors for human gene therapy is unnecessary and could even be inappropriate and potentially dangerous (Kordower & Olanow, 2008). Other authors, however, defend the need for regulatable promoters whenever possible, because they allow continuous monitoring of transgene expression and impede the occurrence of incidents (Cress, 2008). There is little information on the effects of sustained overexpression (months to years) or nonspecific (neurons and glia) of particular genes, which is especially relevant in the case of proteins such as neurotrophic factors whose receptors are widely distributed throughout the CNS. Therefore, it is foreseeable that in the coming years gene therapy clinical trials will start using regulatable gene therapy (rGT) with regulatable vectors (Cress, 2008), which would adjust transgene expression to its maximum biological efficiency with minimum risk of adverse effects.

Transgene expression is coupled to a transcriptional switch in the regulatable vectors (instead of being under the control of a constitutive promoter) whose activity can be regulated by an exogenous chemical. Several regulatable promoter systems by agents such as tetracycline (tet), rapamycin, mifepristone and ecdysone have been tested in animals. The best known regulatory systems are tet systems, which have shown to be efficient and safe in controlling the expression of transgenes in experimental models of various neurological diseases (Régulier et al., 2003; Blesch et al., 2005; Nuber et al., 2008). Tet systems have been developed that can promote activation (tet-ON) or inactivation (tet-OFF) of gene expression, systems that have been incorporated in lentivirus, adeno-associated virus, first-generation adenovirus or high capacity, and retrovirus. The tet system choice depends on the timing of transgene activation which is preferable in each case. It is better to use tet-off for a sustained expression of the transgene, while the tet-ON might be more suitable for transgene expression in shorter time windows, which would minimize the time of exposure of patients to chemicals not without toxicity.

Besides being a safety mechanism against uncontrolled overexpression, regulation of transgene activity could allow flexibility in the control of the therapeutic response, which is difficult to achieve by other methods. The patients' clinical conditions usually change over the course of the disease (Collier et al., 2007); thereby adjusting the dose of rGT could be key to its long-term usefulness. Since the degree of injury is specific for each patient, the response to gene therapy may vary considerably among different patients, so the inclusion

of a failsafe mechanism (in this case of a regulatable vector) could be critical for the treatment of some patients. Neurodegenerative diseases, with the development of biomarkers, could begin to be treated before the onset of symptoms. A regulated system would enable the development of a single gene construct, whose expression could be adjusted to the changing needs of each patient. In addition, advanced systems are being developed that allow the regulation of multiple transgenes introduced into the same vector and are independently controlled by different inducers (Kumar et al., 2004; Goverdhana et al., 2005). This could lead to the development of a complex rGT, in which multiple transgenes (eg, for several neurotrophic factors) may act synergistically on different therapeutic targets.

Despite the many potential benefits of rGT, its clinical use requires further detailed basic studies. There are challenges to be resolved before rGT can be considered as a safe and efficient therapeutic tool. This is the case of regulatable transgene expression inhibition in off (as tet-OFF), which may have toxic effects even at low sustained expression. The penetration would also have to be optimized in terms of the modulating agent in the target tissue /organ. Finally, more studies are needed to determine whether the immune system can recognize components of the system for regulating transgene expression, which may be particularly relevant in cases in which, as with tet-ON, the expression must be maintained for long periods (as would be the case in the treatment of chronic neurological diseases.)

10. Conclusions

Gene therapy is considered to be one of the most promising approaches to develop an effective treatment for PD, and clinical studies using such technology have progressed rapidly in less than a decade. However, the existence of BBB significantly limits its development, so all gene therapy clinical trials for the treatment of PD have used iGT, which has significant drawbacks. It is becoming increasingly clear that from its initial stage, PD does affect non dopaminergic neuronal populations of different brain centres, inducing symptoms that go far beyond the original motor disorders associated with dopaminergic transmission.

The development of new serotypes of adenoassociated vectors, such as AAV9, and of functionalized nanoparticles, such as pegylated immunoliposomes, polymeric nanoparticles, pegylated nanoparticles, dendrimers, fullerenes, as well as specific transporters specific to the low density lipoprotein receptor family, means that it is now possible to introduce and express gene material in nerve tissue following peripherical administration of the above mentioned vectors (niGT). From this perspective, the greatest extension of the effects of niGT might be particularly useful in patients with widespread central affectation, such therapeutic actions are difficult to achieve with iGT. Current gene therapy approaches for PD use non-adjustable constituent vectors, so that in all cases the transgene expression, once introduced it in the body, escapes any external-non-adjustable gene therapy. Therefore, it is foreseeable that in coming years gene therapy clinical trials for PD will start to use regulatable gene therapy (rGT) which would adjust transgene expression to its maximum biological efficiency with minimum risk of adverse effects.

Having crossed the Rubicon, one can then expect the following challenges, for example, to produce efficient vectors with regulatable promoters, to reduce the transduction of peripheral organs, to target vectors to specific neuronal and glial populations, and to demonstrate the reversal of the disease in animal models of PD. The administration of

therapeutic drugs to treat CNS disorders is a common problem shared by pharmacologists and gene therapists, but the field of noninvasive gene transfer in the CNS is on the verge of an exciting step forward. Animal studies are highly promising and it is likely that, in the future, gene therapy procedures will be useful and safe for use in patients.

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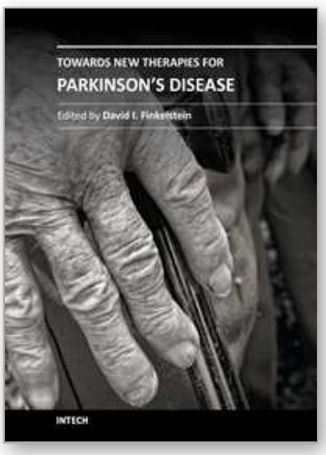

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Parkinson's disease (PD) is characterised clinically by various non-motor and progressive motor symptoms, pathologically by loss of dopamine producing cells and intraneuronal cytoplasmic inclusions composed primarily of ?-synuclein. By the time a patient first presents with symptoms of Parkinson's disease at the clinic, a significant proportion of the cells in the substantia nigra have already been destroyed. This degeneration progresses despite the current therapies until the cell loss is so great that the quality of normal life is compromised. The dopamine precursor levodopa is the most valuable drug currently available for the treatment of PD. However for most PD patients, the optimal clinical benefit from levodopa decreases around five to six years of treatment. The aim of the chapters of this book is to work towards an understanding in the mechanisms of degeneration and to develop disease modifying therapies.

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