Research project overview

1. State-of-the-art & innovative aspects the planned work. The progress of biotechnologies creates new economical challenges in improving the quality of the life by addressing needs related to the health, ageing, food and environment. Gene therapy (GT) opened new perspectives to treat genetic disorders and major pathologies such as neurodegenerative diseases and cancers. The social and economical impact of successful new treatments is universally recognized. The annual cost of neurological diseases is estimated of 100 billion € in the EU and the cost of cancer may be greater than 30 billion € in France alone.

However, in spite of the resources invested in GT, it is still far from a validated medical practice - a modest number of successful clinical trials has been reported. A main reason is that the research on gene vectors has **been unable to address the pharmacological paradigms** due to the lack of tools and approaches to understand the mechanisms of their effects and side effects. For instance, the consequences of gene expression alterations due to integrative vectors were poorly appreciated. The first data on the preferential integration of retroviral vectors into/around transcribed genes came out only in 2002. The clinical trials with SCID patients demonstrated that, while GT vectors offer new medical opportunities, they create new challenges linked to the complexity of viral vectors, which, unlike the classical medicines, involve **unique technological and pharmacological settings**. These challenges must be investigated before using them as new medicines.

To date, the life science research has been performed with limited multidisciplinary approaches and interaction between academia and industry sectors. Public research institutions and biotech SMEs are often in separate environments because joint research/training programmes and cross-mobility of researchers are limited. This may explain the overall productivity of GT in terms of clinical achievements. Therefore, it is necessary that industry and academia modernize their scientific culture in updating the classical biological concepts by integrating new knowledge and approaches. Furthermore, the transversal aspects of research must be present in the mind of researchers to perform translational research, i.e. evolving from pre-clinical studies towards clinical development. Finally, researchers must integrate early in their projects the economic and legal aspects that will facilitate the transition of the innovations towards the industrial sector for their exploitation. Young scientists must also be aware of intellectual/industrial property rights (IPR), understand the importance of upstream/downstream bioprocess development and other transversal issues necessary to transform GT vectors from bench products into new biopharmaceuticals.

n this context, although several vector platforms efficiently deliver coding sequences into the brain, they need to be characterized to be used to treat Parkinson's disease (PD)[1] and other neurological disorders.

The current phase I/II clinical studies using AAV and LV vectors demonstrate a proof-of-concept that therapeutic genes can be delivered to the brain and are tolerated by patients. Compelling evidence has also been accumulated in numerous studies involving toxic models of the disease (MPTP or 6-OH-DA-treatments the induce clinical traits of PD in animals), suggesting that glial cell-derived neurotrophic factor (GDNF) may ameliorate PD symptoms if expressed correctly. Therefore, while results from preclinical experimentation are globally encouraging, as well as the phase I/II trials, a comparative study on gene transfer into CNS has never been performed to establish the pharmacological properties (efficiency and bio-safety) of three viral vectors (AAV, CAV-2 and LV) in preclinical experiments.

[1] This syndrome stems from the loss of neurons confined to the *substantia nigra* accounting for severe motor deficits, with different avenues that affect potentially the course of PD.

2 Objectives. *BrainVectors* will implement the academia-industry interaction by integrating complementary skills between 4 SMEs and 7 academic institutions in 7 countries by comparing gene transfer into CNS with AAV, CAV-2 and LV vectors and assess their therapeutic efficiency and bio-safety in animal models for PD.
The global objectives are:

a) Establish, among the three viral vectors that carry an inducible expression cassette, which yields optimal therapeutic effect.

b) Understand the immune response of these vectors in appropriate models.

S & T objectives: We bring together experts from fundamental virology, neurology, immunology and professionals on bioprocesses and biotechnologies to identify therapies for PD. The overall above objectives are distributed into complementary and intertwined approaches and methodologies:

- Engineer AAV, CAV-2 and LV vectors containing inducer-transactivator couples to deliver neutrophic factors
- Use high-throughput transcriptional analysis to better understand the effects of interaction of these vectors with brain cells
- Assess the immunogenicity of vectors and transgenes with the high-throughput technologies

3. Approaches and methodologies.

3.1 Why use AAV, CAV-2 and LV. Each viral vector has its specific advantages and drawbacks such as cloning capacity, memory or induced immunity, toxicity, specificity, safety, titre, or efficacy. However, there is no single gene transfer vector that can be used, or even imagined, to treat all neurodegenerative disorders. If one wanted to genetically modify neurons the vector should preferentially transduce these highly differentiated cells, which make up ~10% of the primate brain. To date, AAV, CAV-2 and LV have led to long-term transgene expression in CNS with various extents. However, data generated independently make comparison challenging. In *BrainVectors*, we will assay these vectors simultaneously by using the same inducible expression system.

3.1.1 AAV vectors, derived from primate Parvoviruses, are efficient, versatile and safe tools for both biological research and the development of gene therapy. Vector design and production methods have been developed to a point where many proof of efficacy have been obtained in animal models, and clinical trials have been initiated. A dozen different AAV species with differences in their surface properties exist and exhibit variable tropism. This natural diversity can be further engineered using mutagenesis or *in vitro* evolution techniques followed by *in vivo* selection of enhanced tissue specificity. Thus, AAV vectors have the advantage to reach and modify their target with high efficiency and the relative simplicity, versatility and manufacturability. Their drawbacks are the limited size possible of the transgene and the fact that, as all vectors derived from viruses that infect humans, it may trigger immune responses. Notably, reactivation of anti-AAV memory T cells may have been responsible, in part, for the destruction of AAV-transduced liver cells during a Phase I trial. Several AAV-based clinical trials for the treatment of PD were already made according to 3 different strategies by i) complementing neurons with enzymes involved in the dopamine production such as the aromatic L-amino acid decarboxylase, ii) inhibiting the subthalamic nucleus by supplying glutamic acid decarboxylase, and iii) protecting/regenerating dopaminergic neurons by neurturin, a neurotrophic factor. Early encouraging feasibility and tolerability have been reported with these approaches. However the therapeutic efficacy of these trials is still to be demonstrated.

3.1.2 CAV-2 vectors: The advent of helper-dependent human adenovirus (HAd) vectors in the late 1990, has led to nontoxic, highly concentrated and highly homogenous preparations. Ad vectors also poorly integrate into the host genome, yet lead to long-term expression in many tissues in immunologically naïve animals. The large cloning capacity (>30 kb) also allows subcloning an entire gene including transcriptional regulatory elements. These advantages make helper-dependent vectors

ideal for long-term gene transfer to post-mitotic cells like neurons. However, the most obvious drawback of the HAd is that the memory immunity of virion proteins compromises safety and reduce gene transfer efficacy. The majority of humans are not immunologically naïve to these ubiquitous pathogens. Indeed the repeat exposure by multiple HAd serotypes leads to cross-reacting cellular and humoral memory immunity in >95% of the subjects. The anti-virion humoral response might explain in fact, in addition to other factors, the variability between the Phase I trials using HAd vectors. Furthermore, a long-lived proliferative CD4+ T cells can cross-react with multiple serotypes even if the patients had not serological evidence of prior infections, suggesting the presence of common antigenic viral epitopes, and that alternative human serotypes may have limited advantages. In this context, we pioneered the development the canine AdV (CAV-2) as possible solution, because nonhuman virus-derived vectors may be void of certain disadvantage. The development of this new vector system is now in progress with other colleagues, in the frame of the FP7 RTD project *BrainCAV*. Indeed, CAV-2 vectors circumvent the ubiquitous human anti-HAd memory immune response and they are thus capable of long-term neuron-specific expression (i.e. >1 yr in rat brains). Furthermore, multiple injections of vectors amplify (5-fold increase) the transgene expression in terms of transduced dopaminergic neurons in rat *substantia nigra*. Concerning the immune response they showed that CAV-2 do not mature dendritic cells, which are responsible for adaptive and memory immune response need.

3.1.3 LV vectors: The development of LV vectors based on HIV-1, EIAV, or foamy virus, their optimization and the development of highly efficient manufacturing protocols (tri- or quadri-transfection of 293 cells followed by purification) leading to **highly purified** vector preparations, makes these vectors a powerful tool for research, pre-clinical and clinical applications. In addition, improved bio-safety has been obtained recently by generating non-integrative LV vectors and insulators-equipped vectors. Both approaches may reduce eventual effects of the vector promoter(s) on the cellular genes, leading thus to a reduction or elimination of risks associated with insertional mutagenesis. In addition, the use of insulators may protect transgene cassettes from silencing and positional effects. Along the successive development of 3 generations of LV packaging systems[2], **improved biosafety**. With a packaging capacity of ~9 kb, these vectors may be suitable for the treatment of PD.

Efficient gene delivery into CNS has been demonstrated for both HIV-1 and EIAV vectors for at least 8 months. Other experiments include neuroprotection of *substantia nigra* dopaminergic neurons in various PD animal models by using GDNF, and other rescue approaches, such as for retinitis pigmentosa, mucopolysaccharidosis type VII and amyotrophic lateral sclerosis. A clinical trial for the treatment of PD used three key dopamine biosynthetic enzymes (tyrosine hydroxylase, aromatic L-aminoacid decarboxylase, and GTP cyclohydrolase 1) to create a source of dopamine in the region where the neurotransmitter is deficient because of the death of dopaminergic neurons in the *substantia nigra*. Early encouraging feasibility and tolerability have been reported also for these trials However, the efficacy of these trials is still to be demonstrated.

Therefore, *BrainVectors* will characterise AAV, CAV-2 and LV vectors in view of their pharmacological development for the treatment of PD, which may be extended to other neurodegenerative disorders. Indeed, ***vectors with inducers/transactivator couples*** will be generated. Although encouraging results have been generated (they avoid continuous expression of the *tet*-transactivator to maintain the un-induced state), the exogenous transactivator factors (chimeric proteins containing bacterial and viral elements) may provoke immune reactions and hepatic toxicity. Another important issue is the **nature and dose of the inducer**. It is necessary to identify inducers that have lower toxicity. Secondly, the transactivator must be sensitive to the inducer to reduce the required dose to acceptable levels. For example, doxycycline (*dox*), a tetracycline analogue, is preferred to tetracycline because it is already used in the clinic for the treatment of bacterial infections in the brain. Thus, a good inducer should be a compound able to by-pass the blood-brain barrier at very low doses.

[2] The 1st generation LV packaging system encompasses all HIV-1 genes besides the envelope. The 2nd generation LV packaging system is additionally deleted in all viral auxiliary genes, i.e. *vpr*, *vif*, *vpu* and *nef*. The 3rd generation LV packaging system (Dull et al. 1998) comprises only *gag* (virion structural proteins), *pol* (retrovirus-specific enzymes) and *rev* (post-transcriptional regulator for nuclear export and expression of *gag* and *pol* mRNA). The requirement for the *tat* gene has been abolished by placing a constitutive chimeric LTR promoter upstream of the vector transcript.

AAV, CAV-2 and LV vectors with such inducer-transactivator couples will be generated to assay regulated the gene expression in the brain. **(a)** The efficiency of AAV-tetON-M2 vectors/*dox* inducer couple have been assessed and validated in rats. **(b)** New *tet* derivatives will be screened and tested to identify the compounds with the best activity/toxicity ratio in rats. **(c)** Transactivators responding at the lowest inducers' doses will be incorporated into the autoregulatory tetON AAV vectors. The first candidates are inducible at **100-fold lower *dox* dose**. New derivatives will be isolated and screened with the feedback from the immunologist and on the basis of their inducibility in the rodent brain. Notably, the cells expressing the transactivator and the transgene will likely influence the toxicity and immunogenicity of the rtTA derivatives. It will be very important to obtain vectors with a restricted pattern of transgene expression in the brain. We has already derived a liver-specific AAV-tetON vector. With a similar strategy and with the help of other partners expert in gene transfer in the brain, *BrainVectors* will develop brain-specific tetON promoters. The cellular and regional tropism of the new promoters in the rat brain will be analyzed using well-established methods.

3.2 Functional induction transcriptional assays: we will incubate vectors harbouring tet-inducible cassettes with: **(i)** the "classic" primary cultures of human midbrain neurons and glia, **(ii)** a 3D neurosphere suspension culture of neurons and glia, and **(iii)** *in vivo* in the rodent brain. These environments are escalating levels of complexity for the cell-cell contact. In primary cultures the cells interact essentially with the coating on the plastic dish. In the 3D suspension cultures, the neurons and glia form spheres and tight cell-cell interactions. There are no optimal substitutes for the complex cellular interaction that occur *in vivo*.

We will also use transcriptional analysis of AAV, CAV-2 and LV interaction with brain cells. All virions induce some degree of signal transduction during attachment and internalisation. How brain cells deal with the transient signal is unknown. There are several options and approaches that can be used to address the outcome of virion attachment, internalisation, trafficking and transgene expression in brain cells. We chose a global transcriptional or "gene-chip" analysis. Global transcriptional assays may also lead to a comprehensive way to evaluate vector toxicity. This approach is feasible, has high information to cost ratio and it is state-of-the-art. Several transcriptional analyses have been performed and it is now an opportune time for our studies because the initial pitfalls have been identified, data acquisition is straightforward, data interpretation is more informative, and the results are more readily exploitable. Our genome-wide analysis will provide a means to gain further knowledge in this field, especially if complemented with *in vivo* data and pathway analyses. We will therefore compare the response of brain cells to AAV, CAV-2 and LV vectors in our transcriptome assay.

The principle validation techniques, e.g. qRT-PCR and Luminex assays for mRNA and protein quantification, are state-of-the-art. Previously some of these approaches, under our conditions and relative to our data mining protocols, were reliable indicators of genuine biological effects. The flow cytometry approach using intracellular staining for proteins adds another level of refinement to our analysis. The flow cytometry field is exploding with technical advances and the enormous information we will obtain will be integrated into the overall understanding of the brain cell response to viral vectors.

In conclusion, microarray studies can contribute to the understanding of viral biological properties. Statistical meta-analysis, together with the analysis of biological paths modulated by vectors, will be invaluable complementary tools to comprehensively clarify the processes that viral vectors unchain in the host. This information has two main relapses in vector development: the ability to predict the functions altered by vectors independently from the transgene effects, and the possibility to act on these effects with tools aimed at improving efficacy and reducing toxicity. Ideally, we will exploit the data for the identification of virus and/or host factors implicated in possible adverse effects on the biology of neurons. Our data could lead to strategies designed to manipulate the host response through RNA interference for vector improvement.

3.3 Immunogenicity. Understanding the immune response to vectors is crucial to establish their pharmacological characteristics. One reason for the delay of the developmental pathway of GT is that the **immunology of gene vectors is poorly understood** despite the increasing interest of the scientific community and the progress of recent immunotechnologies. For example, when a highly immunogenic $CD8^+$ epitope of the rtTA transactivator (rtTA186CTL), is mutated into expected low or no immunogenic version, it leads to the appearance of other subdominant immunogenic epitopes, which were low

immunogenic before the mutation. This implies that it is necessary to identify the mechanisms triggering the immune response in both normal and diseased tissues/organs for each antigenic component of a given vector. Then one must develop the appropriate **epitope modification strategy**, rather than envisage immunosuppression protocols that induce toxic consequences in patients. Immune rejections have been documented also against AAV-transduced cells. In the brain, little or no immune response has been reported. Nevertheless, preclinical studies have shown that antibodies directed against AAV capsid appeared post-injection and preclude a second administration of vector. Notably, the latter experiments were performed in healthy brain, and the immune response towards AAV and transgenes is still unknown in diseased brains.

Therefore, *BrainVectors* will address the responses against capsid proteins, tet transactivators and therapeutic transgene (i.e. human GDNF) in PD animal models. In addition, variants will be screened by human epitope prediction programs to select those with reduced immunogenicity. Double humanized mice for the common MHC class I and II haplotypes (HLA-A2, -A24, -B7 and HLA-DR) that cover >90% of humans will be used to dissect T and B cell restricted molecular et cellular immune responses against the recombinant vector. The HLA-humanized mice will be either pre-treated with MPTP to simulate the PD pathology and then injected with CAV-2, LV or AAV-tetON vectors. Because the presence of immunodominant epitope may alter the CTL responses against the whole construct produced artificially by the presence of human GDNF in humanized MPTP-treated mice expressing the mouse counterpart, in a parallel study, a HLA humanized mouse model will be generated in which the mGDNF is replaced by its human counterpart, to avoid any alteration between human and mouse GDNF expression; then, we will focus first on the immunogenicity of the rAAV and tet transactivator without including human GDNF. Human GDNF, as a self protein, should not induces an immune reaction in the targeted human situation. Additionally, humoral and CTL responses against the whole construct including human transgene will be addressed in rodent. If important immune responses to the tet transactivator are observed, the transactivator coding sequence will be modified by introducing single amino acid changes, which reduce/abolish the immunogenicity of these epitopes. The HLA-restricted immunodominant epitopes identified in HLA-humanized mice will serve directly for the sensitive and specific monitoring (e.g., using tetramers and ELISPOT) of peripheral CTL responses in PD patients undergoing gene therapy trials.