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Viral Vectors in Neurobiology: Therapeutic and Research Applications

Renata Coura

*Centre de Neurosciences Paris Sud – CNPS – Université Paris Sud XI
France*

1. Introduction

1.1 History and definition of viral vectors

Viruses are intracellular parasites with simple DNA or RNA genomes (Figure 1A). Three steps compose virus life cycle: infection of a host cell, replication of its genome within the host cell environment, and formation of new virions (Figure 1B). This process is often but

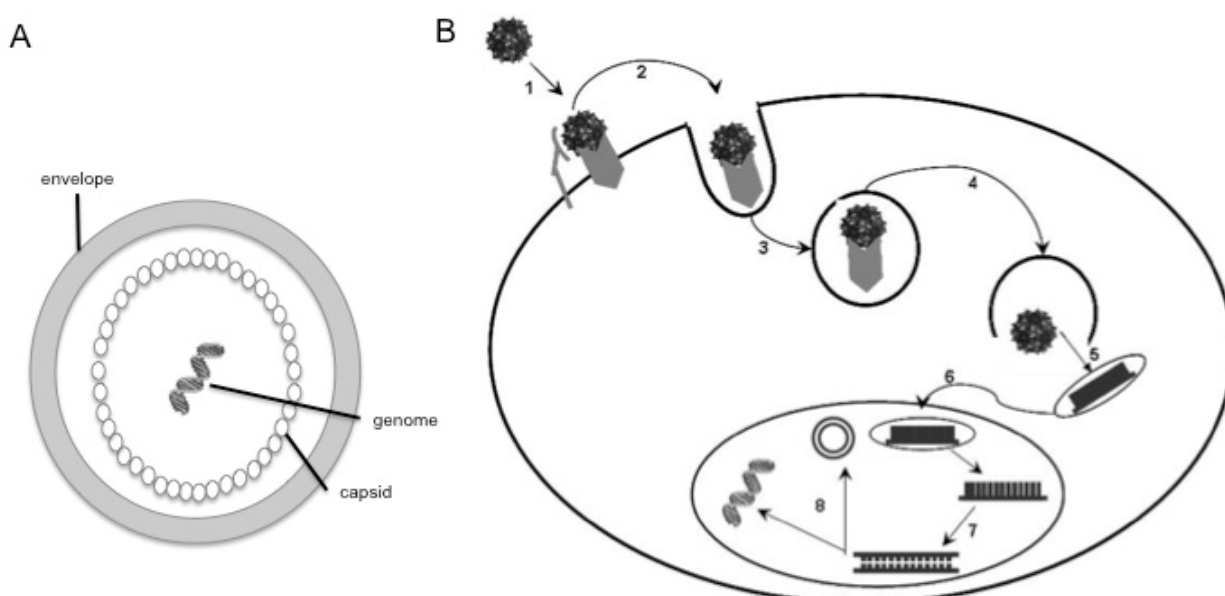


Fig. 1. Virus. (A) Structure. Simplified scheme of virus structure, with a lipid envelope that can be present or not; a protein-composed capsid and the genetic material, that can be DNA or RNA, double or single strand. (B) Life cycle. Example of the course of adeno-associated virus (AAV) productive infection. Scheme showing the eight steps of AAV transduction of host cells: (1) viral binding to a membrane receptor/co-receptor; (2) endocytosis of the virus by the host cell; (3) virus intracellular trafficking through the endosomal compartment; (4) escape of the virus from the endosome; (5) virion uncoating; (6) entry into the nucleus; (7) viral genome conversion from a single-stranded to a double-stranded genome; and (8) integration into the host genome or permanence of an episomal form capable of expressing an encoded gene (from Coura and Nardi, 2008).

not always associated with pathogenic effects against the host organism. Nevertheless, since the mid-1980s, a likely useful role for virus has been envisaged. The idea is to use the unique virus capacity to enter the cell and to replicate their genome to construct vectors, containing the viral envelope and a recombinant genome, so that these vectors could be able to deliver genetic material into cells. Then, recombinant viral vectors are created in which genes essential for viral replication are removed and a gene of interest is inserted in the viral genome (Figure 2). While this eliminates pathogenicity due to viral replication, retention of viral genes and continued expression of these genes may limit the potential of the current generation of vectors. Meanwhile, defective viral vectors represent a different approach, in which only viral recognition signals are used to allow packaging of foreign DNA into a viral coat while eliminating the possibility of viral *gene expression* (see **glossary**) within target cells. These viral vectors would be able to long-term gene delivery to mammalian cells, without pathogenicity and with minimal associated toxicity. Today, several viral vector systems are close to achieving this aim, providing stable transgenic expression in many different cell types and tissues.

1.2 Viral vectors

For the production of an efficacious and safe viral vector it is required at first to identify the crucial viral sequences for viral particle assembly, for viral genome package, and for *transgene* (see **glossary**) delivery to target cells. Then, dispensable genes are deleted from viral genome in order to reduce its pathogenicity and immunogenicity. At last, residual viral genome and transgene are integrated into the construct (Figure 2). Some viral vectors are able to integrate host genome unlike others that remain in an episomal form. Integrative vectors, like retroviruses and adeno-associated vectors are able to promote a persistent transgene expression. Otherwise, non-integrative vectors, like adenovirus whose viral DNA is maintained in an episomal form into infected cells, lead to a transient transgene expression. Each vector presents specific advantages and limitations that become more or less proper depending on the objective of its application (<http://cmbi.bjmu.edu.cn/cmbidata/therapy/research/re02/021.htm>; Osten et al., 2007; Howarth et al., 2010).

The ideal vector has not been described yet, but its characteristics should include:

- Easy and efficient production of high titers of viral particle;
- Absence of toxicity to target cells and undesirable effects like as immune response against the vector or the transgene;
- Capacity of site-specific integration, allowing a long-term transgene expression, as in cases of genetic disorders;
- Capacity of transduction of specific cell types;
- Infection of proliferative and quiescent cells.

Most vectors used for gene delivery are derived from human viral pathogens that have been made nonpathogenic by deleting essential viral genes. They usually have a broad tropism; therefore they can infect and deliver their encoded transgenes to a wide spectrum of cells and/or tissues.

Currently, the most efficient and commonly used viral vectors are adenovirus (Ad), adeno-associated virus (AAV), herpes simplex virus type 1-derived vectors (HSV-1), and retrovirus/lentivirus vectors (Table 1).

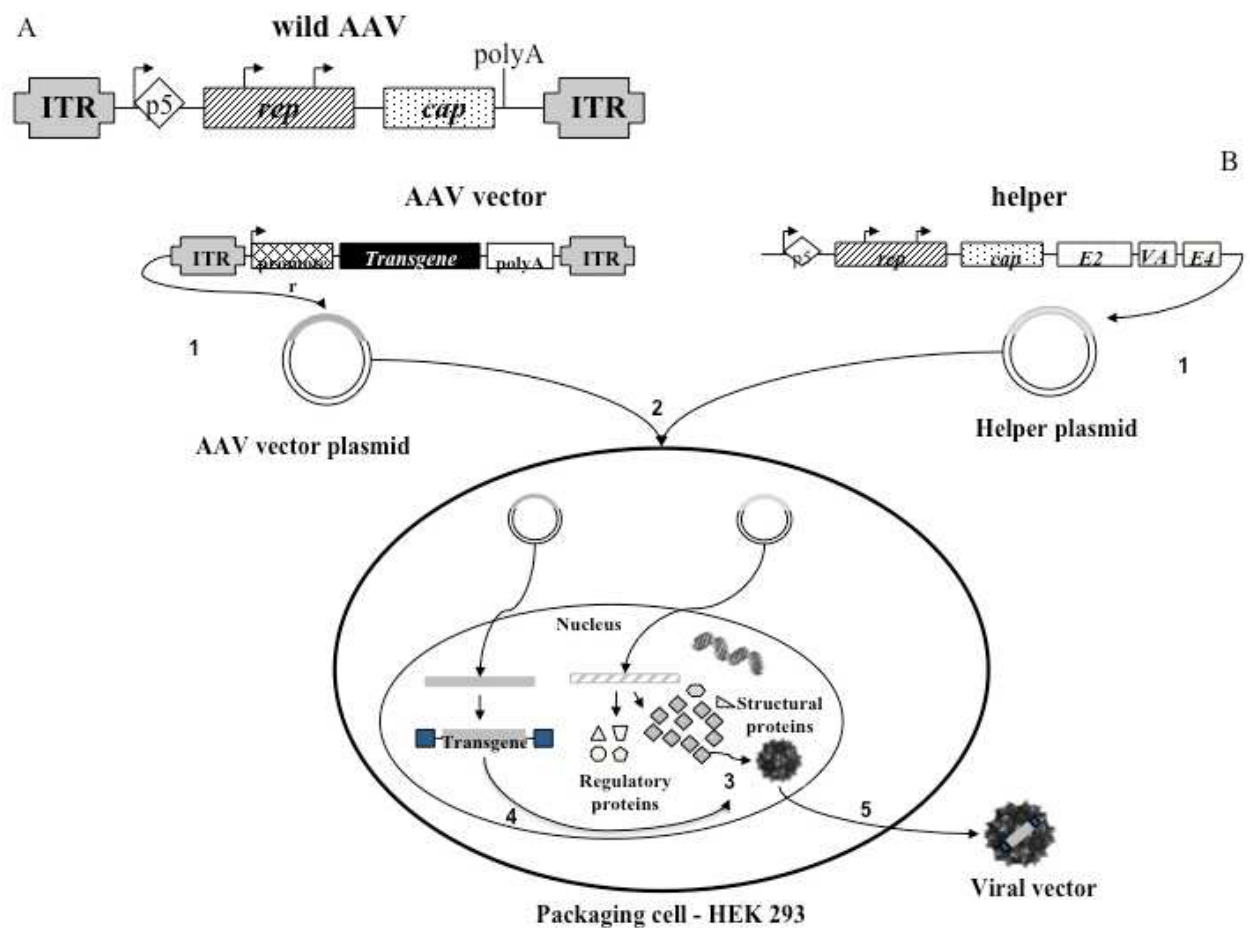


Fig. 2. Example of vector construction. (A) Wild AAV. (B) AAV recombinant vector production. AAV vector cassette, containing only the ITRs of the wild virus and the transgene and its promoter; helper cassette, containing the AAV rep and cap genes required for virus packaging and the Ad genes E2, VA and E4, required for virus replication. (1); co-transfection of both plasmids in HEK 293 packaging cells, whose genome contains the Ad E1 gene, which together with the other Ad required genes, supplied by the helper plasmid, allows the establishment of a productive infection (2); empty capsid is formed by AAV structural proteins assembly into the nucleolus (3); the replicated ssDNA viral genome with the transgene flanked by ITRs (vector plasmid) are packaged into the empty capsid in the nucleoplasm (4), giving rise to a non-replicative recombinant AAV vector virion (5). The regulatory proteins act especially in the replication and packaging processes (from Coura and Nardi, 2008).

Virus vector	Description	Advantages	Limitations	Applications
Adenovirus (Ad)	<ul style="list-style-type: none">• Icosahedric• Non- enveloped• Genome capacity of 36 Kb• Entry into the cell by receptor binding• Episomal (non-integrative)• Capable of transducing postmitotic cells• Persistence of expression for weeks or months• Difficult production• Toxicity	<ul style="list-style-type: none">• Easy propagation in high titers(10^{10}-10^{13});• High transduction efficiency;• Infection of the majority of cell types;• Possibility of insertion of large DNA fragments (up to 8kb);• Infects both replicating and differentiated cells.	<ul style="list-style-type: none">• High immunogenicity, inducing an important cellular and humoral immune response that can be fatal;• Transient expression• Viral proteins can be expressed in host following vector administration	Therapies that require transient gene expression: cancer therapy, angiogenesis induction and DNA vaccine production (due to its inflammatory and immunogenic properties);
Retrovirus (Retrovirus and Lentivirus)	<ul style="list-style-type: none">• Globular• Enveloped• Genome capacity of 8 Kb• Entry into the cell by fusion• Integrative• Capable of transducing proliferative (retrovirus and lentivirus) and quiescent (lentivirus) cells• Persistence of expression for years• Easy production• Minimal toxicity	<ul style="list-style-type: none">• Low immunogenicity;• Possibility of insertion of modest DNA fragments (up to 8 Kb);• High transduction efficiency;• Integrates into host genome resulting in sustained expression of vector• Extremely well studied system• Vector proteins not expressed in host	<ul style="list-style-type: none">• Insertional mutagenesis (random integration)• Low titers (106- 107)• <i>In vivo</i> delivery remains poor. Effective only when infecting helper cell lines	<ul style="list-style-type: none">• Genetic diseases of T cells and hematological diseases (Retrovirus);• HIV/AIDS• Optogenetics• Neuronal tracing
Adeno-associated virus (AAV)	<ul style="list-style-type: none">• Icosahedric• Non- enveloped• Genome capacity of 4.7 Kb• Entry into the cell by receptor binding• Integrative• Capable of transducing postmitotic cells• Persistence of expression for years• Difficult production• No toxicity	<ul style="list-style-type: none">• Low immunogenicity;• Easy propagation in high titers;• Infection of the majority of cell types;• Long-term gene expression	Limited capacity for DNA fragments insertion	<ul style="list-style-type: none">• Genetic Diseases• Tumors• Neurological Diseases• Ocular Diseases• Cardiovascular Diseases• Others

Herpes Simplex Virus (HSV)	<ul style="list-style-type: none">• Icosahedric• Enveloped• Genome capacity of 30 Kb• Entry into the cell by fusion• Episomal (non-integrative)• Capable of transducing postmitotic cells• Persistence of expression for weeks• Difficult production• Toxicity	<ul style="list-style-type: none">• Large insert size• Could provide long- term CNS gene expression• High titer	<ul style="list-style-type: none">• System currently under development• Current vectors provide transient expression• Low transduction efficiency• Toxicity	<ul style="list-style-type: none">• Neuronal gene transfer, <i>in vitro</i> and <i>in vivo</i>.
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Table 1. Main viral vectors systems and their characteristics.

1.2.1 Adenovirus

Adenoviruses (Ads) are medium-sized (90–100 nm), non-enveloped icosahedral viruses composed of a nucleocapsid and a double-stranded linear DNA genome. The viral genome is large, consisting of a single double-stranded DNA molecule 36 to 38 kilobases in size. Viral DNA replication and transcription are complexes, and viral replication and assembly occur only in the nucleus of infected cells. Mature virions are released by cellular disintegration.

There are 55 described serotypes in humans, which are responsible for 5–10% of upper respiratory infections in children, and many infections in adults as well.

Adenoviral infection is initiated by the virus binding to the cellular receptor. Internalization occurs via receptor-mediated endocytosis followed by release from the endosome. After endosomal release, the viral capsid undergoes disassembly while moving to the nuclear pore. Nuclear entry of the viral DNA is completed upon capsid dissociation, and the viral DNA does not integrate into the host genome but remains in an episomal state.

Since adenoviral replication depends on the E1A region of the viral genome, all recombinant adenoviral vectors have this region of its genome deleted, and are referred to as "replication-deficient" virus. Such vectors are capable of infecting a cell only once, no viral propagation occurs, and the infected cell does not die as a result of vector infection. As replication-deficient viruses are required, the 293 cell line is utilized for vector production. This is a human kidney cell line which has been stably transfected with the E1A region of the adenoviral genome. This allows the vector to be made and matured within the 293 cell, yet vectors prepared from this cell line will lack the E1A region and remain replication-deficient.

The non-integrative feature of adenovirus limits their use in basic research, although adenoviral vectors are occasionally used in *in vitro* experiments. Their primary applications are in gene therapy, especially oncolytic gene therapy, and vaccination (Ayuso et al., 2010).

1.2.2 Retrovirus

Retroviruses are globular enveloped virions ranging in diameter from 80 to 130 nm. The viral genome is encased within the capsid along with the proteins integrase and reverse transcriptase. The genome consists of two identical positive (sense) single-stranded RNA molecules ranging in size from 3.5 to 10 kilobases. Following cellular entry, the reverse transcriptase synthesizes viral DNA using the viral RNA as its template. The cellular machinery then synthesizes the complementary DNA which is then circularized and inserted into the host genome. Following insertion, the viral genome is transcribed and viral replication is completed. The majority of retroviruses are oncogenic although the degree to which they cause tumors varies from class to class.

As the inserted vector, called a provirus, remains in the genome, it passes on to the progeny of the cell when it divides. However, the site of integration is unpredictable, what can represent an obstacle in using of these vectors. The provirus can disturb the function of cellular genes and lead to activation of oncogenes promoting the development of cancer (insertional mutagenesis), which raises concerns for possible applications in gene therapy.

Retroviral vectors can either be replication-competent or replication-defective, even if replication-defective vectors are the most common choice. Replication-competent viral vectors contain all necessary genes for virion synthesis, and continue to propagate themselves once infection occurs. Because the viral genome for these vectors is much lengthier, the length of the actual inserted gene of interest is limited compared to the possible length of the insert for replication-defective vectors.

In addition to insertional mutagenesis, the primary drawback to use of retroviruses, such as the Moloney retrovirus, involves the requirement for cells to be actively dividing for transduction. As a result, cells such as neurons are very resistant to infection and transduction by retroviruses.

Lentiviruses are a subclass of Retroviruses. They have been adapted as gene delivery vehicles thanks to their ability to integrate into the genome of non-dividing cells, which is a unique feature of Lentiviruses as other Retroviruses can infect only dividing cells. Moreover, studies have shown that lentivirus vectors have a lower tendency to integrate in places that potentially cause cancer than other retroviral vectors.

For safety reasons lentiviral vectors never carry the genes required for their replication. To produce a lentivirus, several plasmids are transfected into a so-called packaging cell line, commonly HEK 293. One or more plasmids, generally referred to as packaging plasmids, encode the virion proteins, such as the capsid and the reverse transcriptase. Another plasmid contains the genetic material to be delivered by the vector. It is transcribed to produce the single-stranded RNA viral genome and is marked by the presence of the ψ (psi) sequence. This sequence is used to package the genome into the virion (Mátrai et al., 2010; Kumar & Woon-Khiong, 2011; Yi et al., 2011).

1.2.3 Adeno-associated virus

Adeno-associated viruses (AAV) belongs to the genus Dependovirus and family Parvoviridae. It is currently not known to cause disease. The Parvoviridae family is characterized by small, icosahedral and non-enveloped virus whose genome is a single

stranded DNA. AAV is one of the smallest viruses with a capsid of approximately 22 nm and one of the most spread of this family, leading to seropositivity in more than 80% of human population (serotype 2). Despite this high seroprevalence, the virus has not been linked to any human illness, causing a very mild immune response. Because a co-infecting helper virus is usually required for a productive infection to occur, AAV serotypes are ascribed to a separate genus in the Parvoviridae family designated Dependovirus.

The wild AAV has a linear single-stranded DNA genome of approximately 4.7 Kb of either plus (sense) or minus (anti-sense) polarity. The AAV2 DNA termini consist of a 145 nucleotide-long inverted terminal repeat (ITR) that forms a characteristic T-shaped hairpin structure. These ITRs are important in the site-specific integration of AAV DNA into a specific site in chromosome 19. The ability of wild-type AAV to selectively integrate into chromosome 19 made them an attractive candidate for the production of a gene therapy vector that could do the same. The ITRs act as DNA replication origin, as well as signal for package and integration. In addition, they also act as regulator element for wild AAV gene expression. The ITRs flank the two viral genes *rep* (replication) and *cap* (capsid) encoding nonstructural and structural proteins, respectively. The virus does not encode a polymerase relying instead on cellular polymerase activity to replicate its DNA (Ni et al., 1998).

Adeno-associated vectors are prepared by replacing the capsid genes with the gene of interest. Construction of AAV vectors consists of the recombinant AAV vector plasmid DNA, and a non-rescuable AAV helper plasmid, which encodes for the AAV capsid proteins. Also required is either wild-type adenovirus or HSV and cell line for viral propagation. Unlike the previous vector systems described, the cell line need not contain any portion of the AAV genome since all required AAV genome elements could be provided by the two plasmids. Cells are first infected with the wild-type adenovirus or HSV, and then both the recombinant AAV vector plasmid DNA and the non-rescuable AAV helper plasmid are co-transfected into the cells. The cells produce mature recombinant AAV vectors as well as wild-type adenovirus or HSV. The wild-type adenovirus or HSV is removed by either density gradient centrifugation or heat inactivation.

These vectors have been designed to produce a gene therapy vector with site-specific integration and the ability to infect multiple cell types. Unfortunately, this has not been the case to date for these vectors. Current research focuses on how to regain the site-specific integration sequences into the recombinant vector. These vectors do offer some advantages over other vector systems which include the lack of initiating an immune response, their stability and ability to infect a variety of dividing and non-dividing cells. Unfortunately, they cannot incorporate genes larger than 5 kb and must be closely screened for adenoviral or HSV contamination (Berns, 1996; Grimm & Kay, 2003; Coura & Nardi, 2007; Coura & Nardi, 2008; Mezzina & Merten, 2011).

1.2.4 Herpes simplex virus

Herpes simplex viruses are members of the herpes virus family, Herpesviridae, that infect humans. They are ubiquitous neurotropic and neuroinvasive viruses that persist in the body by becoming latent and hiding from the immune system in the cell bodies of nerves. Latency is defined as a state in which viral DNA is maintained within the cell nucleus in the absence of any viral replication. The structure of herpes viruses consists of a relatively large double-

stranded, linear DNA genome encased within an icosahedral capsid, which is wrapped in a lipid bilayer envelope. The HSV genome has been entirely sequenced and is rather extensively studied. As a result, currently, a general knowledge exists of which genes and DNA sequences may be deleted and at which sites foreign DNA may be inserted into the viral genome. These studies also have defined the minimal requirements for viral replication and packaging.

Vectors derived from Herpes simplex virus (HSV) have some unique features. The vectors have a wide host range and cell tropism, infecting almost every cell type in most vertebrates. In addition, the natural property of the virus to infect and establish latent infection indefinitely in post-mitotic neurons has generated substantial interest in using it to deliver therapeutic genes to the nervous system.

The two main strategies for HSV-based vectors in use today are genetically-engineered viruses and plasmid derived "amplicon" vectors. The first strategy involves the construction of recombinant viruses containing deletions in one or more viral genes whose expression is essential for viral replication, resulting in replication-incompetent vectors.

The second strategy involves the use of plasmid-derived vectors containing HSV-1 origins of DNA replication and DNA packaging signals that enable multiple copies of the vector genomes to be packaged into helper virus virions. Helper viruses can be either recombinant viruses containing a deletion within an essential viral gene or viruses containing temperature-sensitive mutations that prevent replication at 37° C (normal body temperature). In the case of the former, the replication of the helper virus and packaging of the amplicon vector DNA must occur in a cell line capable of complementing the mutations in the helper virus.

Regardless of the vector system used, two primary goals must be achieved to enable long-term gene expression in neuronal cells. The first goal involves the construct of mutant vectors which themselves are noncytotoxic to cells. Several studies have noted active expression of a foreign gene by HSV vector constructs, which subsequently became inactivated. Reasons for this are not completely apparent, but evidence suggests that the inactivation is a result of cytotoxic effects induced by vector systems.

The second goal involves designing stable, active promoters capable of expressing appropriate levels of the foreign protein. The specific promoter involved in individual therapies may change according to the type, status and activity of the neuronal cell of interest.

Currently, many studies have demonstrated long-term stable transgene expression in the nervous system. In addition, preclinical studies on models of neurological disease, such as glioma, peripheral neuropathy, chronic pain and neurodegeneration, show encouraging results (Jenkins & Turner, 1996; de Silva & Bowers, 2009; Glorioso & Fink, 2009; Manservigi et al., 2010; Fraefel et al., 2011; Goins et al., 2011).

1.3 Viral vectors in neurobiology

Gene transfer into the central nervous system (CNS) shows great promise for basic and clinical research in neurosciences. As the brain presents a high level of structural complexity, it is a complicated target to be accessed and for genetic manipulation.

Currently, viruses are the most widely used vehicles for gene transfer into the adult mammalian brain. However, there does not exist a "universal ideal vector" and each basic

or clinical approach may require a specific set of technical hurdles to overcome. Several viral vectors have been studied. Each one has shown great advantages and disadvantages depending basically on the subset of target cells and the specificities of each research or clinical indication.

The number of suitable vectors for basic research surpasses those being used in clinical trials. Currently, the most widely used vectors in neurobiology are AAV, HSV and lentiviral vectors. AAV presents several serotypes with specific cell tropism. AAV serotype 2 (AAV2), for example, infects neurons preferentially, but seems to not infect all types of neurons equally well. Other serotypes, as AAV4 and AAV5, show distinct tropism and diffusion properties. The construction of vectors combining more than one serotype and using specific cell promoters, allows genetic manipulation of specific sets of neurons, with more sustained and effective transgene delivery and expression. AAV vectors are highly effective for gene delivery and are non-toxic. The main limitation of these vectors is its relatively small gene capacity (McCown, 2011).

HSV is neurotrophic and shows a highly efficient retrograde and anterograde transport within the CNS, being able of entering in a benign latent state. HSV vectors have a large transgene capacity and can assure long-lasting transgene expression. However, the main disadvantage of this type of vector is its cell toxicity and low transduction efficiency. Currently, other variants that try to surpass these limitations have been developed.

Lentivirus vectors have a modest packaging capacity, induce minimal immunological response and can produce long-term transgene expression. In addition, envelope-engineered vectors can show broad cell tropism. On the other hand, these vectors show poor *in vivo* delivery and present the risk of insertional mutagenesis. However, as lentiviruses mostly transduce terminally differentiated cells, the risk of insertional mutagenesis is less important than observed for other retroviruses (Kaplitt & Pfaff, 1996; Davidson & Breakefield, 2003; Howarth et al., 2010; Snyder et al., 2010).

2. Therapeutic and research applications

Viral vectors were originally developed as an alternative to *transfection* (see **glossary**) of naked DNA for molecular genetics experiments. Compared to traditional methods such as calcium phosphate precipitation, *transduction* (see **glossary**) can ensure that nearly 100% of cells are infected without severely affecting cell viability. Furthermore, some viruses integrate into the cell genome facilitating stable expression.

Protein coding genes can be expressed using viral vectors, commonly to:

1. Increase concentration of a certain protein and study its function (over-expression studies);
2. Antagonize function of a certain protein (expression of dominant negative proteins and RNAi constructs);
3. Make the cell produce fluorescent indicator proteins (for example, EGFP or Ca²⁺ sensitive proteins). These may be used to monitor various variables within the living cells (tracing and *in vivo* imaging);
4. Control neuronal excitability using light-sensitive ion channels (optogenetics);
5. Pre-clinical and clinical gene therapy approaches.

Therefore, viral vectors constitute an important tool and present extraordinary opportunities for basic and clinical research, particularly in neurosciences.

2.1 Gene expression *in vitro* and *in vivo*

Viral vectors have emerged as an important tool for manipulating gene expression in the adult mammalian brain. The adult brain is composed largely of non-dividing cells, and therefore DNA viruses have become the vehicle of choice for neurobiologists interested in somatic gene transfer. By re-expressing an absent protein or by overexpressing a certain protein (i.e increasing its concentration), one can unravel its functions and possible process that it underlies (Howarth et al., 2010).

2.2 Tracing

Detailed knowledge of the complex anatomical interconnections of the central nervous system (CNS) plays an important role in the understanding of brain function, both in physiological and in pathological conditions. For this, *in vivo* tracing of neural tracts constitutes an important tool. Some viral vectors systems, especially lentivirus, are largely used to this end. The idea is to construct vectors expressing a marker protein, like GFP that are anterogradely (from the cell soma to the *neurites* - see **glossary**) or retrogradely (from the neurites to the cell soma) transported along the neurons (Figure 3). In this manner, one can inject an anterograde vector into a certain brain region in order to see where its projections are issued. Similarly, using a retrograde vector, one can identify from where come its inputs (Figure 4) (Masamizu et al., 2011).

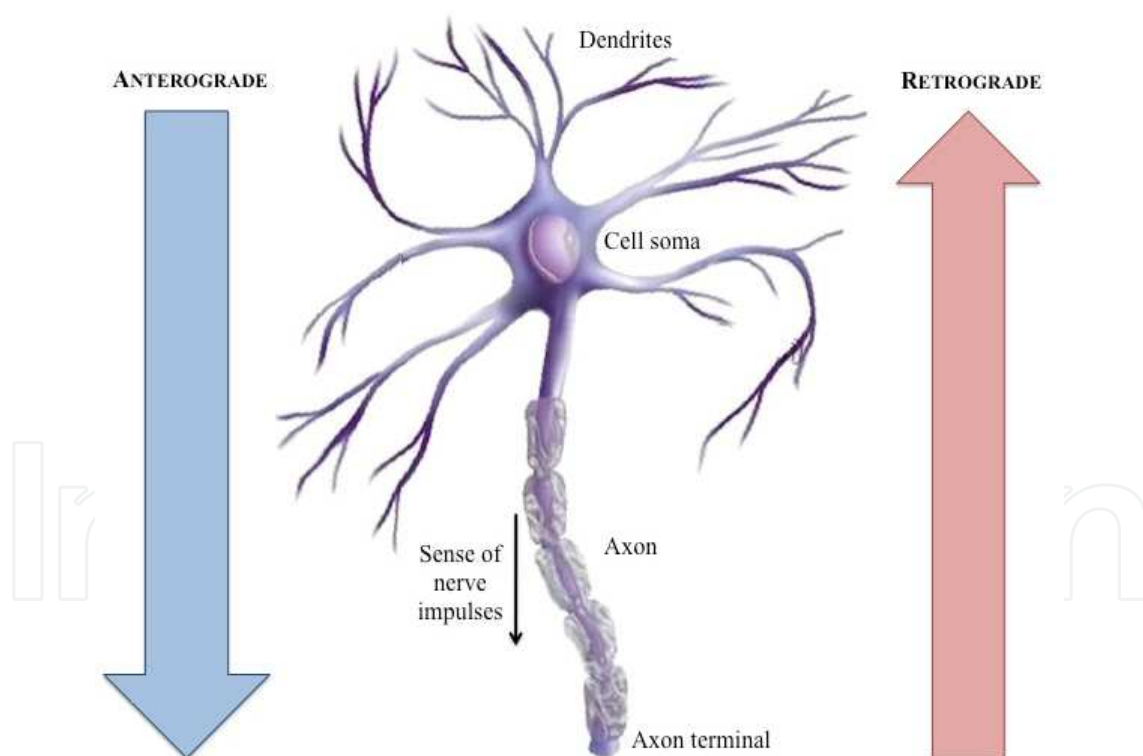


Fig. 3. Transport anterograde and retrograde. The former is from the cell soma to the axon terminals. This is also the sense of the nerve impulses. Conversely, retrograde transport is from the axon terminals to the cell soma. Then, if one injects an anterograde vector in a certain brain region, we will observe to where it issues its projections. On the other hand, injection of a retrograde vector, allows us to know from where come the inputs of the injected brain region.

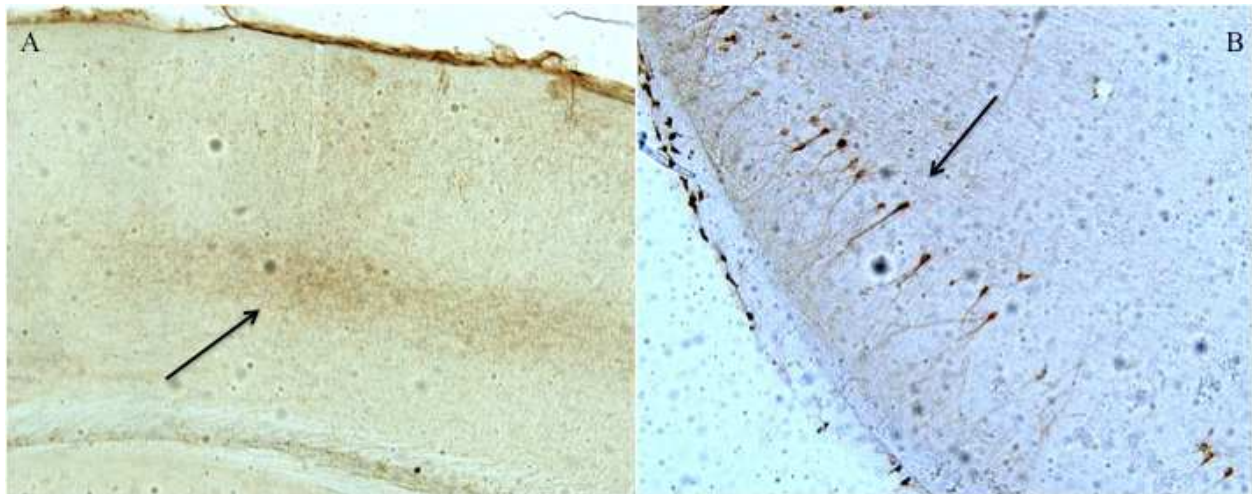


Fig. 4. (A) Optical microscopy of an anterograde vector, showing axon projections from the prefrontal cortex. (B) Optical microscopy of a retrograde vector, showing cell soma that inputs into the prefrontal cortex.

2.3 Optogenetics

Optogenetics is a new and promising gene and neuroengineering tool, which allows the control of the activity of defined populations of neurons. With this technology, by the combination of genetic and optical methods, imaging or control of specific events in target cells of living tissues with temporal precision became possible. The optical part consists in the use of specific light-sensitive proteins expressed in the neurons of interest. These are channel proteins that have either been rendered responsive to light or that are inherently light-sensitive, making possible the manipulation, that is, activation or inhibition, of neuronal activity.

The genetic methods concern to the construction of *expression cassettes*, using cell type specific *promoters* (see **glossary**). Specifically coupling the DNA sequence of the light-sensitive protein with a cell-specific promoter, one can drive protein expression in neurons defined by a common genetic identity and, possibly, common functional roles.

Optogenetics has provided a tool that may overcome some of the limitations of traditional neuromodulation techniques. Activation or inhibition of specific neuronal populations with different wavelengths of light opens up possibilities for modulating neural circuits with previously unimagined levels of precision.

Optogenetics have advanced rapidly since 2005, when it has been demonstrated that neurons expressing the ChR2 (channelrhodopsin-2) protein can generate action potentials when illuminated by light with a specific wavelength (Boyden et al. 2005). This protein is derived from a unicellular alga that was engineered for stable membrane expression into mammalian brain. This is a light-sensitive cation channel that triggers *depolarizing action potentials* (see **glossary**) when exposed to 470 nm blue light. Currently, ChR2 protein variants have been generated in order to improve its functionality, especially concerning deactivation kinetics and activation lasting.

Another light-sensitive protein largely used as an optogenetics tool is a bacteria-derived chloride pump, called halorhodopsin (NpHR). This protein provokes neuronal activity

inhibition, by generating a fast chloride ion influx whereby it leads to membrane *hyperpolarization* (see **glossary**), when activated by 570 nm yellow light. As for ChR2, more efficient variants of NpHR have been generated.

As ChR2 and NpHR proteins are activated by separate wavelengths of light, activation or inhibition of action potential activity in ChR2- and NpHR-expressing cells can be independently controlled. Moreover, co-expression of ChR2 and NpHR within a single neuron allows bidirectional control of membrane action potential.

These light-sensitive proteins, both belonging to the family of opsin genes, can be genetically targeted into specific brain cell types using stereotactically injected viral vectors. Lentiviral and adeno-associated viral (AAV) vectors are both suitable for such gene transfer. Notably, AAV has been considered a safer vector for CNS gene transfer and has been used in several clinical trials, because of its innocuous and non-pathogenic features.

Other expression systems were developed that are more suitable for basic and preclinical researches than for clinical trials. This includes generation of transgenic mice expressing ChR2 in a subset of neurons. Another approach consists in the use of Cre-recombinase *knock-in mice* (see **glossary**) injected with Cre-activated AAV vectors encoding opsin genes (Henderson et al., 2009; Fiala et al., 2010; Knöpfel et al., 2010; Mancuso et al., 2010; Kravitz & Kreitzer, 2011; LaLumière, 2011; Yizhar et al., 2011).

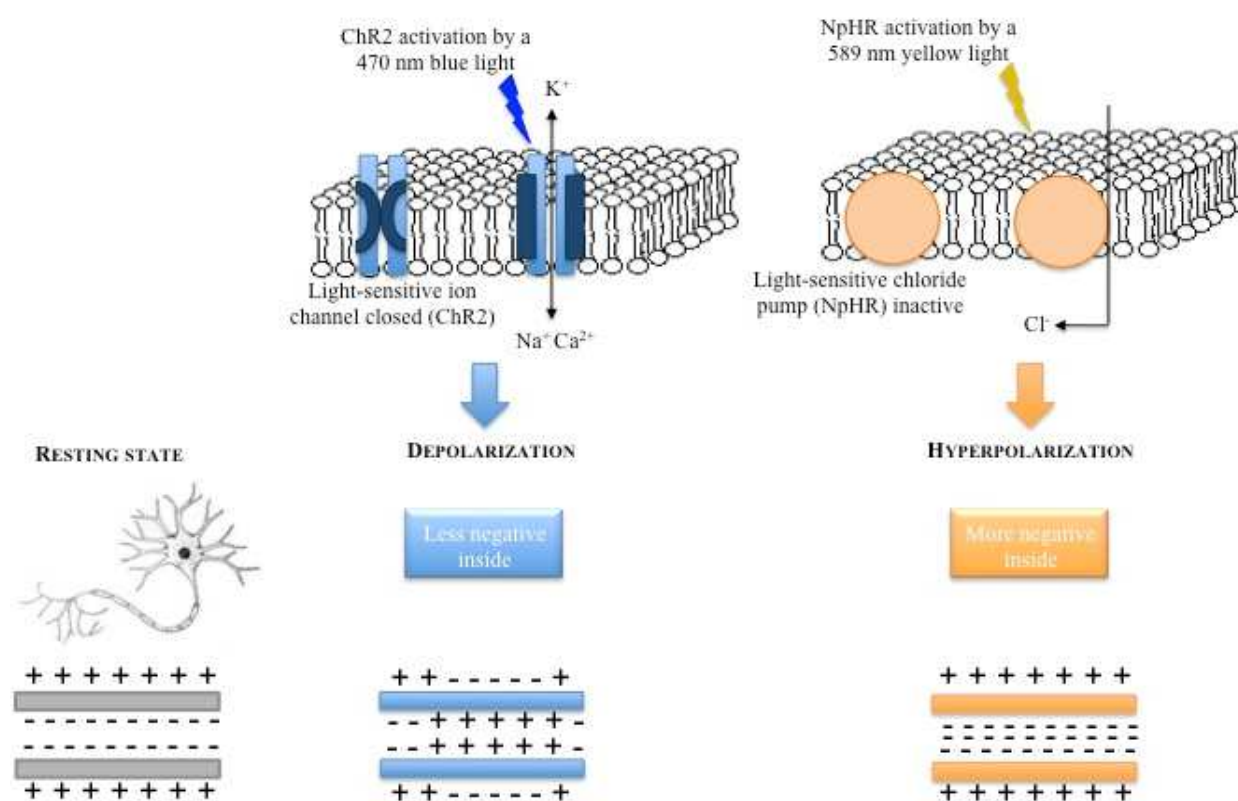


Fig. 5. Sensitive-light proteins and the basis of optogenetics technology.

These techniques have been used in the analysis of neural circuits, activation of reflexive behavior, induction of behavior plasticity, linking cell activity to behavior, among others. As an example, using a combination of Cre-inducible ChR2 with parvalbumin-positive

interneuron-specific or pyramidal cell-specific Cre driver cell lines, it was identified that parvalbumin fast-spiking interneurons have a specific and important role in the generation of gamma-oscillations in the barrel cortex and that perceptual decisions and learning can be controlled by a subset of excitatory neurons, in mice (Cardin et al. 2009; Sohal et al., 2009).

Currently, the possible application of optogenetic tools with therapeutic approaches for neurological conditions just starts to be considered and investigated. Possible targets would be activation and recovery of breathing, suppression of seizure-like activity, amelioration of parkinsonian symptoms, recovery of blindness and optical deep brain stimulation. Yet, peripheral nervous system targets could also be plausible (Nagel et al., 2005; Gradinaru et al., 2009; Adamantidis et al., 2010; Dani et al., 2010; Gradinaru et al., 2010; Zhang et al., 2010; Depuy et al., 2011; Kokaia & Sørensen, 2011; Peled, 2011; Tønnesen et al., 2011).

2.4 Gene therapy

Gene therapy (GT) constitutes a therapeutic intervention based on modifications of the genetic material of target cells, by either correcting genetic defects or overexpressing therapeutically useful proteins. Initially designed to definitely correct monogenic disorders such as cystic fibrosis, severe combined immunodeficiency or muscular dystrophy, gene therapy has evolved into a promising therapeutic modality for a diverse array of diseases. Targets are expanding and currently include not only genetic, but also many acquired diseases, such as cancer, tissue degeneration or infectious diseases. Over the past years, significant progress was made concerning to *enabling technologies* (see **glossary**), molecular understanding of several of these diseases and manufacturing of vectors. The basic prerequisites to GT success include therapeutically suitable genes, appropriate gene delivery systems and proof of safety and efficacy. Among them, gene delivery systems constitute a crucial requirement. Depending on the duration planned for the treatment, type and location of target cells, and whether they undergo division or are quiescent, different vectors may be used, involving non-viral methods, non-integrating viral vectors or integrating viral vectors. Advances, especially, in viral vectors engineering and improved gene regulatory systems to facilitate and tightly control therapeutic gene expression have leading to GT progress (Coura & Nardi, 2008).

The first gene therapy clinical trial was carried out in 1989, in patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral transduction. In the early 90's, a clinical trial with children with severe combined immunodeficiency (SCID) was also performed, by retrovirus transfer of the deaminase adenosine gene to lymphocytes isolated from these patients. Since then, more than 5,000 patients have been treated in more than 1,000 clinical protocols all over the world (<http://www.wiley.co.uk/genetherapy/clinical>). Despite the initial enthusiasm, however, the efficacy of gene therapy in clinical trials has not been as high as expected; a situation further complicated by ethical and safety concerns (Coura & Nardi, 2008). Further studies are being developed to solve these limitations.

2.4.1 Brain gene therapy

The advances of modern molecular biology and *in vivo* gene therapy have challenged neuroscientists with the potential prospect of genetically manipulating post-mitotic neurons. The ability to alter gene expression in these cells would open the door towards potential therapies for several disorders such as Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis. Gene therapy using viral-based vectors has received

considerable attention and represents a major focus of ongoing research. Viral vectors using several different human viruses such as adeno-associated viruses, herpes viruses and lentiviruses are currently being developed for brain gene therapy purposes. Gene therapy directed towards neuronal cells however, presents unique problems. These problems include the genetic manipulation of post-mitotic (*i.e.*, non-dividing) cells, the ability to specifically infect neurons, long-term maintenance of the vector DNA and expression of the target gene within the neuronal cells. Herpesviruses, particularly herpes simplex virus type 1, have unique characteristics of infection, replication and pathogenesis which make them potentially ideal candidates for the development of viral vectors capable of altering endogenous gene expression or delivery of foreign genes both *in vivo* and *in vitro*.

In the same way, AAV vectors present great potential and appear to be much more promising for a wide range of gene therapy approaches. Current studies have been leading to great improvements in AAV vector and expression cassette design and novel AAV serotypes have been identified, that have improved AAV vectors efficacy (Kaspar et al., 2002; Coura & Nardi, 2007; McCown, 2011).

Vectors based on minimal self-inactivating (SIN) and pseudotyping lentiviruses have been considered as relevant vector for research and clinical applications. In these vectors, the entire coding regions of the virus are removed and provided in *trans* from separated expression cassettes, so that they present less than 1 kb of viral genome and express only the transgene cassette. In addition, the use of an envelope from another virus (pseudotyping), like the vesicular stomatitis virus (VSV-G), can give vectors broad species and tissue tropism. Moreover, innate and adaptive immune responses against these lentiviral vectors are not significant. All together, these features are encouraging their use for CNS applications (Escors & Breckpot, 2010).

All these vectors have been largely used in pre-clinical and/or clinical trials. In many indications, both AAV and lentiviral vectors are being assessed. This is the case for gene therapy approaches in epilepsy, multiple sclerosis, Alzheimer disease, diabetes, Parkinson's disease, chronic pain, lysosomal storage disorders, amyotrophic lateral sclerosis, brain ischemia, seizure, Huntington disease, and others. HSV vectors have also been used for some of these indications, but currently, they have been frequently applied for gene therapy approaches in brain tumors, as is the case for adenovirus vectors as well (Maingay et al., 2005; Wong et al., 2006; Robinson et al., 2007; Lowery et al., 2009; Shih et al., 2009; Björklund et al., 2010; Manfredsson & Mandel, 2010; Eskenazi & Neumaier, 2011; Jacobs & Wang, 2011; Van der Perren et al., 2011; Vande Velde et al., 2011; Thaci et al., 2011).

3. Summary

Viruses are intracellular parasites with simple DNA or RNA genomes with a unique capacity to enter the cell and to replicate their genome. This has given rise to the possibility to construct vectors containing the viral envelop and a recombinant genome, so that these vectors could be able to deliver genetic material into cells. Viral vectors hold promise for basic and clinical research in neurosciences and currently the vehicles of choice for gene transfer into the adult mammalian brain. Main viral vectors exploited for use in neurobiology are adeno-associated vectors, herpes simplex vectors and lentiviral vectors. Each has both advantages and disadvantages depending basically on the subset of target cells and the specificities of each research or clinical indication.

The spectrum of research and therapeutic applications of viral vectors increase to the extent that advances are made in this area. Currently, these applications include functional studies by gene expression *in vitro* and *in vivo*; neuronal tracing, allowing unraveling anatomical neuronal interconnections; neuromodulation, using optogenetic tools and gene therapy.

Nevertheless, for each approach there are still a lot of technical hurdles to overcome. However, advancement in this field will most likely lead to new tools being created with the ability to overcome current limitations.

4. Glossary

Term	Definition
Gene expression	The process of formation of messenger RNA (mRNA) of a DNA template which then is translated into the sequence of aminoacids at the ribosome to make proteins.
Transgene	A foreign gene introduced into the cell (for example by a viral vector).
Transfection	Process of delivering a foreign gene into the target cell, by a non-viral vector or naked DNA.
Transduction	Process of delivering a foreign gene into the target cell, by a viral vector.
Expression cassette	A piece of DNA containing elements (promoter, coding part and polyadenylation signal) necessary for expression of a transgene.
Promoter	Region of DNA that facilitates the transcription of a particular gene. Promoters are located near the genes they regulate, on the same strand and typically upstream (towards the 5' region of the sense strand).
Cre	Cre is a 38 kDa recombinase protein from bacteriophage P1 which mediates intramolecular (excisive or inversional) and intermolecular (integrative) site specific recombination between loxP sites. A loxP consists of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region. The Cre's DNA excising capability can be used to turn on a foreign gene by cutting out an intervening stop sequence between the promoter and the coding region of the transgene.
Gene knockout	It is a genetic technique in which one of an organism's gene is made inoperative, basically by gene deletion or disruption.
Knock-in	Genetic engineering method that involves the insertion of a protein coding sequence at a particular locus in an organism's chromosome.
Neurites	Refers to any projection from the cell body of a neuron. This projection can be either an axon or a dendrite.
Depolarization	Change in a cell's membrane potential, making it more positive or less negative. A large enough depolarization may result in an action potential that triggers excitation of the neuron activity.
Hyperpolarization	Change in a cell's membrane potential, making it more negative, inhibiting the rise of an action potential and, consequently inhibiting neuronal activity.
Enabling technology	Set of new processes and new techniques that enable the development and improvement of existing technologies.

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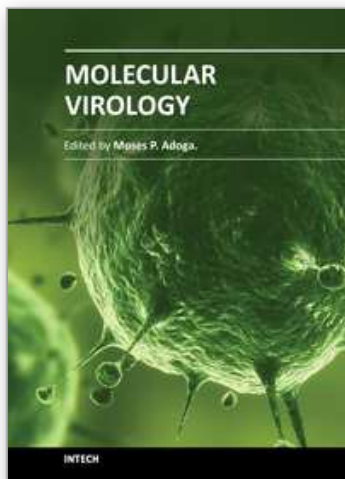
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This book covers various aspects of Molecular Virology. The first chapter discusses HIV-1 reservoirs and latency and how these twin phenomena have remained a challenge to eradication. Aspects regarding the molecular evolution of hepatitis viruses including their genetic diversities with implications for vaccine development are treated in the second chapter. Metabolic disorders that are a consequence of hepatitis C virus infection are discussed in the succeeding chapter. The following two chapters discuss influenza C virus and the applications of viral vectors in therapeutic research. Avian influenza is handled in the sixth chapter and the therapeutic potential of belladonna-200 against japanese encephalitis virus infection is discussed in the succeeding chapter. The last two chapters discuss baculoviruses and their interaction with polydnaviruses. Researchers, lecturers and students will find this book an indispensable companion.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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