Chapter 11

Gene Delivery Strategies

After reading this chapter, you should be able to:

- Describe common methods of delivering recombinant DNA into cells
- Compare and contrast methods of DNA delivery in various in vitro and in vivo preparations

Techniques covered:

- **Physical gene delivery**: microinjection, electroporation, biolistics
- **Chemical gene delivery**: calcium phosphate transfection, lipid-based transfection
- **Viral gene delivery**: adeno-associated virus (AAV), lentivirus (LV), canine adenovirus (CAV), rabies virus (RV)

The previous chapter discussed methods for making and manipulating DNA constructs using recombinant DNA technology. Of course, the ultimate goal of creating a DNA construct is to deliver the manipulated sequence into living cells so that the endogenous cellular machinery can transcribe and translate the sequence into functional proteins. A neuroscientist might want to deliver a DNA sequence into a population of cells in culture, in a brain slice, or in the brain of a living animal. The purpose of this chapter is to survey the common methods of delivering recombinant DNA into cells in each of these environments.

There are three general categories of DNA delivery: physical, chemical, and viral. **Transfection** refers to nonviral methods of delivering DNA to cells, including physical and chemical methods. **Infection** refers to viral DNA delivery, in which viruses attach themselves to cells and inject their DNA cargo. Each method has its own relative advantages and disadvantages that make it particularly well suited for delivering DNA in different experimental contexts (Table 11.1). Major factors that dictate the choice of DNA delivery method include cellular environment, cell type, and experimental goals. Additionally, these DNA delivery methods vary in terms of the number of cells they affect, levels of gene expression they induce, and the length of gene expression over time.

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TABLE 11.1 Categories of Gene Delivery Strategies.

Method	Advantages	Disadvantages
Physical	• High-efficiency gene transfer • No limitations on construct size • No cell type dependency	• Low throughput • Requires specialized equipment • Can physically harm cells
Chemical	• High efficiency in vitro • No limitations on construct size • Relatively easy to perform • Rapid • High throughput • Low immunogenicity	• Limited in vivo applications • Efficiency depends on cell type • Can be toxic to cells • Transient expression
Viral	• High-efficiency gene transfer • Cell-specific targeting is possible • Long-term expression • Can be used in vitro and in vivo • Many viruses delivering commonly used transgenes are available commercially	• Complex cloning required • More expensive than transfection methods • Safety concerns regarding production of infectious viruses in humans • May provoke immune response • Laborious preparation • Limited construct size

PHYSICAL GENE DELIVERY

Physical gene delivery methods deliver DNA into a cell by physically penetrating the cell membrane with force. These methods are often highly efficient (transfected cells express high levels of the delivered gene) and can be used with any cell type. However, they can sometimes disrupt the integrity of the cell membrane, traumatizing the cell, and leading to its death. Therefore, care must be taken in moderating the amount of force applied to deliver DNA to cells. Three common physical methods include microinjection, electroporation, and biolistics.

Microinjection

A scientist can microinject a solution containing DNA into a cell by piercing the cell membrane with a small glass needle and then applying pressure (Fig. 11.1). If the scientist uses an extremely thin needle and withdraws the needle carefully, the cell membrane remains intact, and the cell has an opportunity to incorporate the injected DNA into its genome. This process

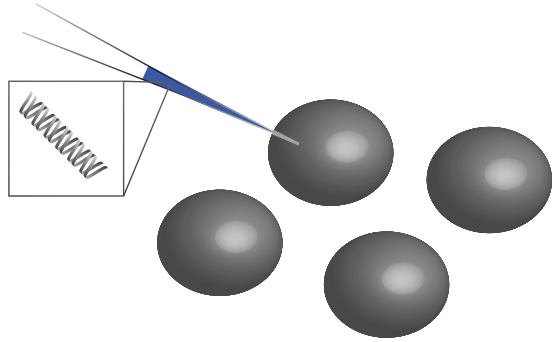

FIGURE 11.1 **DNA microinjection.** An extremely thin glass needle punctures the plasma membrane, allowing DNA to be injected into cells.

requires a microscope to view the living cell, as well as fine micromanipulators to precisely place the needle adjacent to the cell membrane. Each cell must be injected one at a time (in contrast to other methods that deliver DNA to millions of cells at once), making microinjection laborious and low throughput. This method requires technical skill and is therefore usually performed by trained technicians or lab personnel who use these techniques regularly. Microinjection is rarely used to transfect neurons or glia as their small shapes and sensitivity to perturbation make them more difficult to inject than other cell types.

DNA microinjection is the method used to make genetically modified organisms. To make a transgenic mouse, a scientist microinjects a transgenic DNA construct into newly fertilized mouse eggs. In some of the new eggs, the transgene randomly incorporates into the mouse genome. These cells are injected into a female mouse to carry the egg to gestation. The full details of creating transgenic mice, as well as other genetically modified organisms, are discussed in Chapter 12.

Electroporation

Electroporation is the process of using an electric pulse to transfect cells with DNA (Fig. 11.2). Applying an electric field to cells is thought to induce temporary pores in the cell membrane, allowing the cell to take up DNA sequences. The electric field also drives negatively charged DNA strands away from the cathode (negative end) toward the anode (positive end) of an electric field. Therefore, an electric pulse causes some of the DNA to enter the cell. After the electric field is switched off, the cell membrane reseals, trapping some of the electroporated DNA within the cell.

In neuroscience research, electroporation has been particularly useful for in utero preparations in rodents (Fig. 11.3). In utero electroporation surgeries

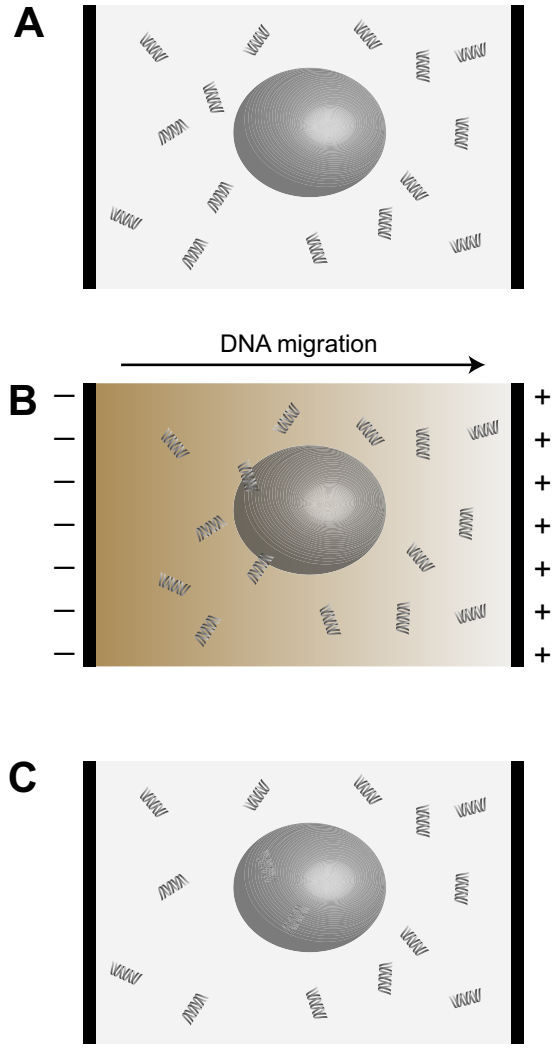

FIGURE 11.2 **Electroporation.** (A) Cells are placed in a special chamber with a solution containing the DNA to be transfected. (B) Applying an electric field increases the permeability of the cell membrane and drives the negatively charged DNA strands away from the cathode and toward the anode. (C) After the electric field is switched off, the cell membrane reseals, and some DNA remains in the cell.

begin by exposing the embryonic pups of a pregnant mother at a precise developmental timepoint. DNA is injected into the pups' ventricles, and electrode paddles direct the uptake of the injected DNA construct(s) toward the anode and away from the cathode. The pups are placed back inside the mother, who is sutured and allowed to recover. Days later, pups are removed or

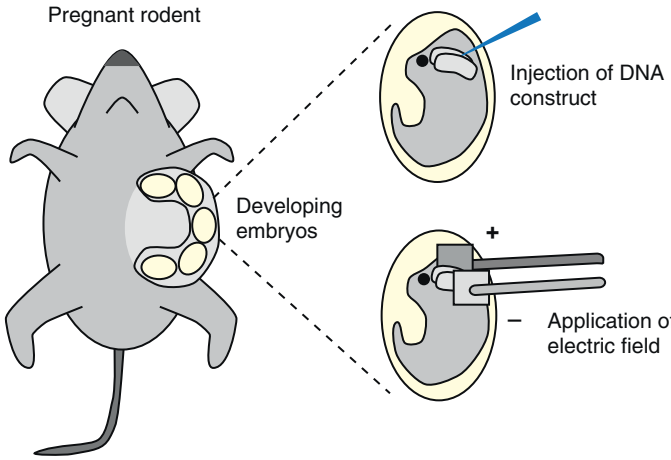

FIGURE 11.3 **In utero electroporation.** A scientist performs a surgical procedure on a pregnant female, exposing the embryonic pups. DNA is injected into the pups' ventricular system, and paddles generate electric field pulses to introduce DNA into the cells lining the ventricles.

the mother gives birth, and the transfected cells of the offspring express the introduced DNA construct. Similar methods can be used in the chicken model system in a process called *in ovo* electroporation. These methods are useful for delivering genes to brain regions adjacent to the nervous system's fluid-filled ventricles, as the DNA-containing solution must be injected into the ventricle. Thus, they have primarily been used in studies of chick spinal cord and rodent cerebral cortex. A scientist can control both spatial and temporal specificity by controlling the position of the electrode paddles and the timing of electroporation. For example, a scientist can target gene transfection to distinct cortical layers in the mammalian cerebral cortex by electroporating at specific embryonic time periods.

Electroporation has numerous advantages, such as the ability to transfect cells in many different in vivo and in vitro environments. Furthermore, investigators can vary expression levels of a transfected gene by varying the strength and pattern of the electric field pulses.

Biolistics

Biolistics, short for "biological ballistics" and also known as **particle-mediated gene transfer**, is the method of directly shooting DNA fragments into cells using a device called a **gene gun**. In neuroscience research, this method has been useful for transfecting neurons in cultured brain slices.

To use a gene gun, a scientist first mixes a DNA construct with particles of a heavy metal, usually tungsten or gold. These fine particles stick to the

negatively charged DNA. The DNA/metal particles are loaded onto one side of a plastic bullet (Fig. 11.4). A pressurized gas, usually helium, provides the force for the gun. Gas pressure builds up until a rupture disk breaks, driving the plastic bullet down a shaft. The plastic bullet is abruptly stopped at the end of the shaft, but the DNA/metal particles emerge from the gun with great speed and force. If the gun is aimed at biological tissue, some of the metal particles will penetrate the cell membranes and deliver DNA constructs to cells.

A neuroscientist can use biolistic technology to cause efficient gene expression in neurons. This technology can produce a dispersed transfection pattern, similar to a Golgi stain, in which only individual cells receive the foreign DNA in a background of untransfected cells. Another advantage to using biolistic technology is that a gene gun can deliver DNA through relatively thick tissue, which is why it is useful for cultured brain slice experiments. This technique has not been successful in transfecting mammalian neurons in vivo, although it has been used in vivo to transfect liver and skin cells. The main disadvantage of using this technology is that it may cause physical damage to cells. Optimization is required to limit the amount of tissue damage caused by the force of impact of the projectiles.

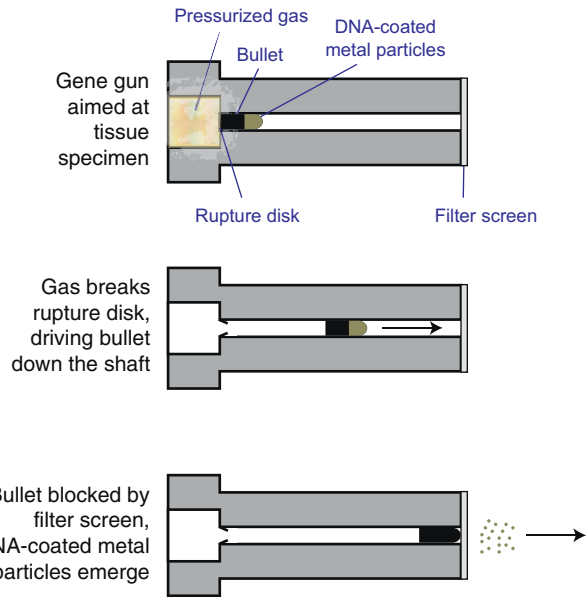

FIGURE 11.4 **A gene gun.** DNA-coated metal particles are placed on the front end of a specialized bullet. High-pressured gas drives the bullet down a shaft. At the end of the shaft, the bullet is blocked by a filter screen, but the DNA-coated particles emerge with great speed and force.

CHEMICAL GENE DELIVERY

Chemical gene delivery is the process by which a scientist uses a chemical reaction to deliver DNA into cells. Negatively charged DNA can form macromolecular complexes with positively charged chemicals. These complexes can then interact with a cell's membrane, promoting uptake through endocytosis or fusion. Chemical gene delivery strategies are useful in that they are incredibly high throughput and often simple to perform. However, they are generally inefficient for in vivo delivery and are therefore mainly used for cell culture experiments (Chapter 13). Expression of the target gene is usually transient, lasting for days to weeks depending on the cell type. Common chemical gene delivery strategies include calcium phosphate transfection and lipid transfection.

Calcium Phosphate Transfection

Calcium phosphate transfection is one of the simplest and least expensive methods of chemical gene delivery in cell culture preparations (Fig. 11.5). This method requires two chemical solutions: calcium chloride, which serves as a source of calcium ions, and HEPES buffered saline (HBS), which serves as a source of phosphate ions. First, the calcium chloride solution is mixed with the DNA to be transfected, and then the HBS is added. When the two solutions are combined, the positively charged calcium ions and negatively charged phosphate ions form a fine precipitate. The calcium ions also cause the DNA to precipitate out of solution. After a few minutes, the solution with precipitate is directly added to cells in culture. By a process that is not entirely understood, the cells take up some of the precipitate, and with it, the DNA. It is thought that the DNA precipitate enters cells by endocytosis, but the exact mechanism remains a mystery. This method works best for a cell monolayer so that the DNA precipitate covers the cells evenly.

The advantages to using the calcium phosphate method are that it is relatively easy, reliable, and cheap. It is useful for transient expression or creating stable cell lines from immortalized cells (Chapter 13). Some immortalized cell lines, such as HEK 293T and HeLa cells, exhibit high transfection efficiencies (90%−100%), which is one reason for their ubiquitous use in the life sciences. The main disadvantage is that transfection efficiencies are low in neurons (1%−3%), and this method does not work at all for transfecting neurons in intact tissue. To transfect neurons in culture, most scientists use lipid-based transfection tools.

Lipid Transfection

Lipofection, also known as "lipid transfection" or "liposome-based transfection," uses a lipid complex to deliver DNA to cells. Lipids are a broad class

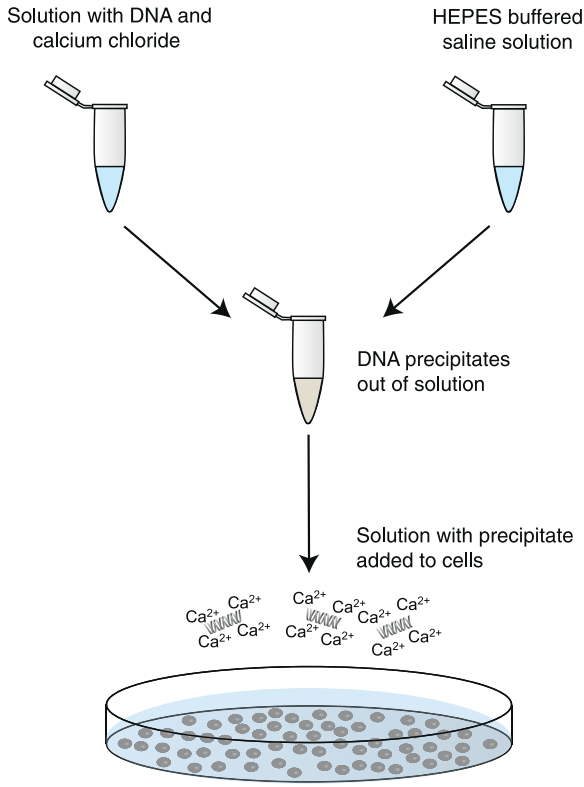

FIGURE 11.5 Calcium phosphate transfection. A scientist mixes DNA, calcium chloride, and HEPES buffered saline. The chemical reaction forms a DNA—calcium phosphate precipitate that is able to pass through the cell membrane and deliver DNA to the nucleus.

of fat-soluble biomolecules, such as fats, oils, and waxes. The cell membranes of animal cells are composed of a bilayer of phospholipids with hydrophilic surfaces facing the cytoplasm and extracellular environment (Fig. 11.6A). Lipofection technology uses tiny vesicular structures called **liposomes** that have the same composition as the cell membrane (Fig. 11.6B). A scientist performs a simple reaction that forms a liposome around the DNA sequence to be transfected (Fig. 11.6C). Depending on the liposome and cell type, the liposome can be endocytosed (Fig. 11.6D), or it can directly fuse with the cell membrane to release the DNA construct into cells (Fig. 11.6E).

The advantage to lipofection is that it works in many cell types, including cultured neurons. Commercially available kits allow transfection reactions to be performed within 30 minutes and gene expression to be assayed within hours. However, like the calcium phosphate method, lipofection is almost exclusively used in cell culture experiments.

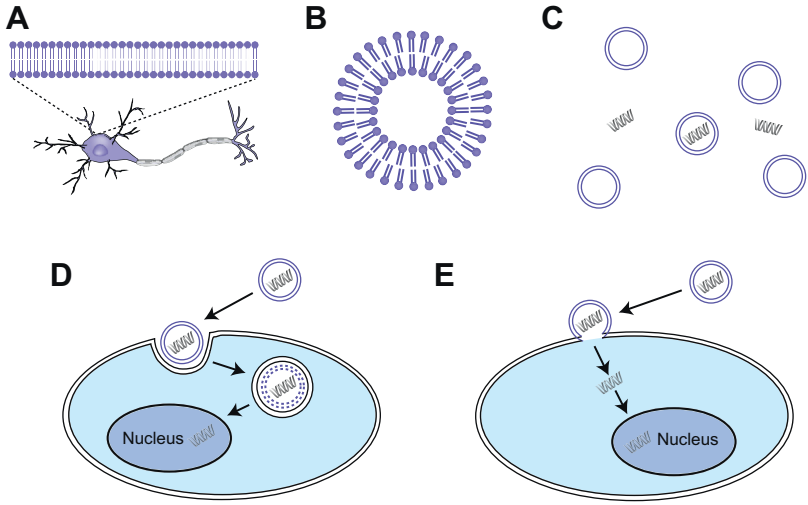

FIGURE 11.6 **Lipofection.** (A) The cell membrane is composed of a lipid bilayer, with a hydrophobic interior and hydrophilic exterior. (B) Liposomes are also composed of a lipid bilayer arranged as a spherical shell. (C) A scientist performs a brief reaction that allows liposomes to form around DNA. (D) Cells in culture can endocytose the liposome, digesting it within vesicles to release DNA. (E) Alternatively, liposomes can directly fuse with the plasma membrane, directly releasing DNA into cells.

VIRAL GENE DELIVERY

Viral gene delivery uses one of many available viral vectors to deliver DNA to cells in vitro or in vivo. A virus can be thought of as a tiny molecular machine whose entire purpose is to attach to cells and inject genetic material. In nature, this genetic material encodes the proteins necessary to make more virus, so the infected cell essentially becomes a virus-making factory. In the lab, many viruses have been manipulated so that they can no longer replicate inside a host cell on their own. Additionally, the coding region of viral DNA is exchanged with a gene of interest to make the virus deliver a transgene to a cell without forcing that cell to produce more virus particles. This process of using a nonreplicating viral vector to deliver foreign DNA into a cell is called **transduction**.

Scientists can either produce virus in their own labs or outsource viral production commercially (Fig. 11.7). To begin, a scientist uses recombinant DNA technology (Chapter 10) to place a DNA sequence of interest into a plasmid containing the necessary sequences to incorporate into a virus particle. Note that this plasmid is not the same as a virus itself but is simply a piece of DNA with the necessary sequences to incorporate into a virus particle, provided the other necessary proteins are present. To produce a batch of virus, cultured cells are used to produce all the necessary viral components. A

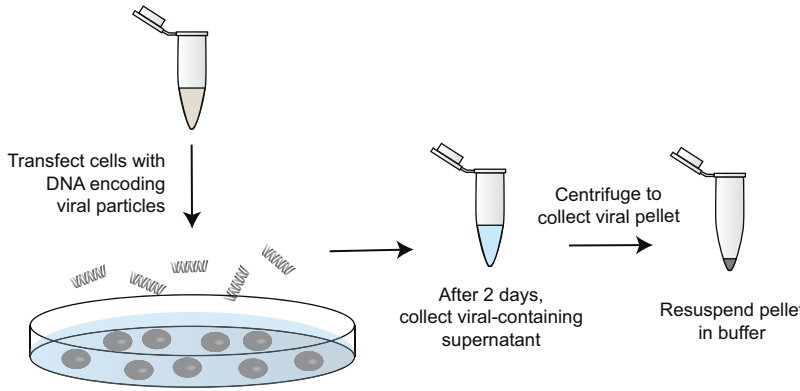

FIGURE 11.7 **Virus production.** A scientist transfects cultured cells with DNA encoding the necessary proteins to make virus. Over 2−3 days, the cells produce virus and release it into the culture medium. The medium is collected and centrifuged to collect a viral pellet. The pellet is resuspended in buffer and frozen until use.

scientist transfects these cells with the recombinant plasmid, as well as additional DNA sequences coding for the necessary viral proteins. Because viral DNA has been engineered so that it cannot replicate more virus on its own, these viral proteins are necessary to produce functional, infectious viral particles. Immortalized cell lines, such as HEK 293T cells, are the best cells to serve as virus packaging cells, as they grow quickly, are transfected relatively easily, and can produce large amounts of virus. 1−2 days after transfection, the **packaging cells** produce many virus particles and release them into the extracellular medium. The final step of virus production is to collect the medium, filter out cell debris, and centrifuge the medium to collect the viral pellet. The pellet is dissolved in a buffer and, depending on the needs of the investigator, either used immediately to infect the target cells of interest or frozen until needed.

Viral infections are robust, highly efficient, and can lead to long-term expression. Viruses have been used to deliver genes to cultured cells, brain slices, tissue explants, and brain regions in vivo. They are the tools of choice to deliver genes into the brains of rodents, as most other methods of gene delivery are incapable of working efficiently in vivo. However, there are also disadvantages and limitations to using viral gene delivery. The infected cells, especially cells in vivo, may not begin expressing the transduced gene at high levels until 7−14 days after exposure to the virus. Also, the size of the viral vector restricts the size of the DNA construct that a scientist can transduce. Finally, scientists must take great care and safety when using viruses, as it is possible for scientists to accidently infect themselves.

There are a variety of viral vectors that are useful to neuroscientists (Table 11.2). They vary in terms of infection efficiency, expression levels,

TABLE 11.2 Viral Vectors Used in Neuroscience.

Vector	Genome Size	Features
Adeno-associated virus (AAV)	5 kb	• Single-stranded DNA virus • Naturally replication-deficient—safe • Infects dividing and nondividing cells • Stable expression • High transduction efficiency • Onset of expression in weeks • Minimal toxicity • Labor-intensive production
Lentivirus (LV)	8 kb	• Double-stranded RNA virus • Stable expression—integrates in the genome • Form of retrovirus that can infect nondividing cells in addition to dividing cells • Safety concerns
Canine adenovirus (CAV)	7.5–30 kb	• Double-stranded DNA virus • Exhibits retrograde transport properties • Infects dividing and nondividing cells • Transient expression—does not integrate in the genome • High transduction efficiency • Onset of expression in days
Rabies virus (RV)	12 kb	• Single-stranded RNA virus • Exhibits retrograde transport properties • Safety concerns • Triggers immune response • Toxic to cells over days and weeks

duration of expression, time to start of expression, host cell toxicity, and host cell preference. AAV and LV are the most commonly used viral vectors due to long-term expression of transgenes and low toxicity in neurons.

Adeno-Associated Virus

Adeno-associated virus is naturally replication deficient, requiring a helper virus to replicate, so it is safer to use than other viral strains. AAV can infect both dividing and postmitotic cells, and causes stable, long-term gene expression. AAV is also less toxic to neurons relative to other viral vectors and does not cause a substantial immune response. However, AAV production is labor intensive and the carrying capacity is relatively small, about 5 kb. AAV is commonly used in a neuroscience research for a variety of research

applications. For example, many labs use AAV to transduce neurons with opsins or DREADDs (Chapter 8) for functional experiments or fluorescent-based activity indicators (i.e., GCaMP, Chapter 7) for imaging experiments. Recently, scientists have engineered novel AAV variants to expand the AAV toolkit. For example, some variants can travel retrogradely (retrograde AAV), while others can cross the blood−brain barrier, allowing for broad and noninvasive targeting of neurons throughout the brain.

Lentivirus

Lentivirus belongs to a class of virus called retrovirus that has an RNA genome rather than DNA. To produce functional gene products, the virus also contains the enzyme reverse transcriptase, which produces cDNA from the RNA template (Chapter 10). When a cell endocytoses a lentivirus particle, the RNA is released and reverse transcriptase produces cDNA. The DNA migrates to the nucleus where it integrates into the host genome.

Most retroviruses only infect dividing cells, making them useful for studying neuronal development and cell fate. However, lentivirus is capable of infecting both dividing and postmitotic cells (such as neurons) and is therefore widely used in neuroscience experiments. Lentivirus is based on the HIV virus and has an 8 kb carrying capacity. Because the DNA integrates into the genome, lentivirus delivery leads to long-term expression.

Canine Adenovirus

Canine adenovirus is a form of adenovirus that is highly infectious in dogs. This virus has a high carrying capacity of 7.5−30 kb, and is used in neuroscience research because of its retrograde properties. Upon injection into the brain, CAV can be taken up by the presynaptic membrane and retrogradely transported to the cell soma. Therefore, this viral vector is useful for targeting neurons based on their connectivity.

Rabies Virus

Rabies virus is an enveloped, single-stranded RNA virus that is primarily used by neuroscientists to map neural circuits in vivo. Once rabies infects a cell and the cell synthesizes new viral particles, those new particles can then enter and infect presynaptic neurons. Scientists have engineered modified rabies viruses to restrict their ability to infect certain neurons as well as their ability to replicate. A modified rabies system has proven useful as a cell-type specific retrograde tracer (see Chapter 6). However, even these modified rabies viruses are quite toxic to neurons due to the high level of viral replication that occurs.

TABLE 11.3 Choosing a Gene Delivery Strategy.

Environment	Considerations	Commonly Used Gene Delivery Methods
Cells in the intact nervous system of living animals	Cells within the brain are notoriously resistant to most transfection methods but can be infected with viral vectors	Virus, electroporation
Cells in brain slices	Gene delivery must be fast and efficient and may need to go through thick tissue	Electroporation, biolistics
Cells in dissociated cultures	Gene delivery must be highly efficient and high throughput to transfect/transduce thousands or millions of cells	Chemical transfection, virus
Individually harvested cells	Cells are often valuable, such as extracted embryos or newly fertilized eggs, so care is taken to efficiently deliver DNA to each cell	Microinjection, electroporation

CONCLUSION

This chapter has surveyed common methods used to deliver DNA sequences into cells. A scientist chooses one method over another based on a number of factors, such as the cell type and the cell's environment, as well as the specific goals of the experiment (Table 11.3). For cell culture experiments, chemical, electroporation, or viral gene delivery strategies all work well. For in vivo DNA delivery to adult animals, viral gene delivery is the most efficient option. Now that we have surveyed these methods, the next chapter will focus on methods of manipulating the genomes of living organisms.

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