Toolbox

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Transfection Techniques for Neuronal Cells

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Introduction

The transfection of nucleic acids into cells is crucial for the study of many aspects of neuronal cell biology. These include investigating gene and protein function by knocking down target proteins via RNA interference (RNAi) or microRNAs, expressing tagged proteins to track their subcellular localization, behavior, and turnover; and expressing mutant versions of proteins to study the functions of specific domains or mimic disease conditions. Moreover, reporter proteins can be used to detect intracellular ion concentrations or levels of gene expression.

Despite efforts to optimize transfection techniques and protocols for neurons, no method has yet been developed that is suitable for all applications. Instead, the various established methods have their own advantages and drawbacks concerning transfection efficiency, expression levels, cell survival, and viability. Other considerations are the ease of use, reproducibility, cost, and applicability to a given experiment. Researchers therefore often face a bewildering roster of possibilities, making it difficult to decide which approach to take.

In this review we provide a brief overview of methods used to transfect mammalian neural precursors and postmitotic neurons either isolated from the developing brain or already established in culture. Like other postmitotic cells, differentiated neurons present a particular challenge regarding the efficiencies for introducing and expressing exogenous constructs. Another important limitation of working with mammalian neurons is that they tend to be very sensitive to physical stress, alterations in temperature, pH shifts, or changes in osmolarity. It is therefore important to manipulate them as carefully as possible during preparation and the transfection procedure.

Because of limited space, our scope is restricted to the most common methods currently used and to important recent advances. We highlight which techniques are especially suited for a given question or context. In particular, we focus on protocols that yield high transfection efficiencies (as is needed for quantitative or biochemical analyses) or minimally perturb cell physiology (an important consideration for live cell imaging). Summaries of the advantages and drawbacks of the different methods and their suitability for a given experiment are summarized in Table 1.

Electrical transfection methods

Electroporation temporarily alters the properties of the plasma membrane by exposing cells to a voltage pulse. This allows charged extracellular material, e.g., plasmids, to enter the cell (Washbourne and McAllister, 2002). Electroporated material mainly enters the cytoplasm. Therefore, the expression rates of transfected plasmids in postmitotic cells, such as neurons, tend to be relatively low with conventional electroporation (up to 15–20%), and high transfection efficiencies are often achieved at the expense of cell survival and viability. Moreover, electroporation generally only works with embryos or dissociated neurons, restricting its use to undifferentiated cells, and there are reports of subsequent developmental abnormalities such as uncharacteristically long neurites. These disadvantages complicate analyses of neuronal differentiation and hamper patch-clamp experiments (Dib-Hajj et al., 2009).

Nucleofection is a modified form of eletroporation, which uses a series of high voltage pulses that enable plasmids to directly enter the nucleus. In addition to cell type-specific transfection programs, nucleofection solutions are used that mimic the physiological microenvironment of the transfected cell type during the procedure. These modifications tend to result in higher transfection rates [e.g., an average of 60–80% after optimization and up to 95% for neuronal cells isolated from embryonic day 17 (E17) rat brains], better cell survival than that afforded by conventional electroporation techniques, and normal subsequent differentiation into mature neurons in culture (Zeitelhofer et al., 2007; Zeitelhofer et al., 2009b).

Nucleofection is the method of choice when high transfection efficiencies are essential, such as for quantitative analyses of knock-down efficiencies after RNAi, where untransfected cells would skew the analyses (Zeitelhofer et al., 2007). While short hairpin RNA plasmids tended to be comparatively difficult to nucleofect with high efficiencies, a recent study has achieved

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Table 1. Summary of advantages and disadvantages of different techniques commonly used to transfect mammalian neurons

	Best suited for	Strengths	Limitations	Toxicity	Onset, level, and duration of expression[a]	Maximum insert size	Genome integration
Electroporation	Cell types/tissues: Neuronal cell lines and freshly isolated primary neuronal cells *in vitro*; whole embryos *in vivo* Applications: Transfection of large numbers of robust cells *in vitro* (in suspension) or *in vivo* when high transfection efficiencies are required	Simple and quick protocol, relatively little optimization required	(1) Can only be used for freshly isolated neurons or cells in suspension that have yet to produce neurites; (2) transfection efficiencies limited by premature voltage pulse termination; (3) relatively expensive equipment and reagents	Variable depending on cell type and electroporation parameters used (robust cell types tend to survive the procedure better)	Typically within hours; expression levels variable depending on electroporation parameters used	No limit	No
Nucleofection	Cell types: Neuronal cell lines and freshly isolated primary neuronal cells *in vitro* Applications: (1) Quantitative and biochemical analyses because of very high transfection efficiencies, e.g., assessment of protein downregulation after RNAi-mediated knock-down; (2) introduction of genetic material into neural progenitor cells with subsequent expansion and/or differentiation	(1) Very high transfection efficiencies (typically ~50%; up to 95% after optimization); (2) often results in nuclear localization of transfected plasmids yielding higher expression rates; (3) reproducible and simple to perform once the procedure has been optimized	(1) Can only be used for freshly isolated neurons or cells in suspension that have yet to produce neurites; (2) relatively expensive equipment and reagents; (3) can require optimization of programs and nucleofection solutions used	Relatively low cell toxicity because of finely regulated sequences of voltage pulses and cell type-specific nucleofection solutions	Typically within hours; moderate expression rates, therefore possible to harvest cells up to several days after transfection to assess, for example, maximal downregulation of target proteins or long-term phenotypes	No limit	No
Single cell electroporation	Cell types: Individual neuronal cells *in vitro* or *in vivo*, including mature, fully differentiated neurons Applications: (1) Transfection of neurons in brain slices and in intact brains of living animals; (2) assessment of the morphology, function, and behavior of single cells in intact neuronal networks; (3) electrophysiological recordings on individual neurons	(1) Surviving neurons are completely functional; (2) subsequent transfection of the same neuron with more than one construct at different time points; (3) neurons up to 1 mm deep into a tissue can be transfected	(1) Relatively time consuming; (2) expensive equipment; (3) relatively difficult to optimize	Moderate cell survival rate	Typically within hours; near physiological expression levels possible because physiological environment is maintained (depending on promoter used), therefore possible to image cells over months	No limit	No
Ca^{2+}-phosphate co-precipitation	Cell types: Neuronal cell lines; differentiating and mature (fully differentiated) primary neurons *in vitro* Applications: (1) Analyses requiring low numbers of transfected cells, e.g., live imaging of individual neurons *in vitro*; (2) analyses depending on healthy neurons such as the assessment of developmental/morphological phenotypes in neurons, e.g. after siRNAi; (3) covisualization of RNAs and proteins	(1) Very cost-effective; (2) no specialized equipment required; (3) comparatively simple to optimize for a variety of plasmids; (4) gentle method with minimal stress for the transfected cells (after optimization); (5) amount of transfected DNA can be titrated to vary expression levels	(1) Low transfection efficiencies for post-mitotic neurons (typically ~5–10%), but can go up to 30% after optimization; (2) transfection procedure can be relatively time consuming (progress of crystal formation and deposition on cells as well as cell viability may have to be monitored regularly over several hours)	Low (when crystal size and exposure time are optimized)	Typically within hours; depending on promoter and cell type: physiological expression levels within the first 12–18 h	No limit	No
Lipofection	Cell types: Neuronal cell lines; differentiating and mature (fully differentiated) primary neurons *in vitro* (and *in vivo* following injection of transfection solution) Applications: (1) Transfection of a wide range of constructs and oligonucleotides; (2) high transfection efficiencies (with little optimization) for cell lines; (3) high efficiencies for RNAi knockdowns, also in mature neurons	(1) Very simple and fast procedure with few optimization steps; (2) suitable for transient and stable transfections; (3) high reproducibility; (4) cost-effective	Relatively low efficiencies for post-mitotic neurons (typically ~1–5%), but can go up to 30% after optimization	Adverse effects on neuronal morphology and/or viability have been reported (depending on cell type and reagent)	Typically within hours; moderate to nearly physiological expression depending on promoter and cell type	No limit	No

(*Table continues.*)

Table 1. Continued

	Best suited for	Strengths	Limitations	Toxicity	Onset, level, and duration of expression[a]	Maximum insert size	Genome integration
Adenoviruses	Cell types: Cell lines and primary neuronal cells, including mature, fully differentiated neurons, *in vitro*; whole nervous system, including adult nervous system, *in vivo* Applications: (1) Efficient *in vitro* and *in vivo* gene delivery, including expression of GOIs only in certain brain regions after localized inoculation with viral vectors; (2) transient and inducible expression possible; (3) suited for quantitative and biochemical analyses because of very high transduction efficiencies	(1) Very high transduction efficiency in dividing and nondividing mammalian cells; (2) no risk of insertional mutagenesis, as there is no genome integration	(1) Labor intensive and expensive; (2) safety issues (biosafety level 2 laboratory needed); (3) immune/inflammatory responses *in vivo*; (4) transduction of glia cells (can be limited with neuron-specific promoters)	High when high virus titers are used	Onset after a few days; high levels of expression that can last for weeks to even months	~7.5 kb (high-capacity, helper-dependent AdVs: up to 34 kb)	No
Adeno-associated viruses	Cell types: Cell lines and primary neuronal cells, including mature, fully differentiated neurons, *in vitro*; whole nervous system, including adult nervous system, *in vivo* Applications: (1) Efficient *in vitro* (cell lines, primary postmitotic neurons) and *in vivo* gene delivery; (2) transient and stable transduction; (3) transduction of neurons in brain slices; (4) suited for quantitative and biochemical analyses because of very high transduction efficiencies; (5) natural tropisms allow specific transduction of different cell types	(1) Very high transduction efficiencies in dividing and nondividing mammalian cells; (2) naturally replication incompetent/non-pathogenic; (3) can integrate into the host genome	(1) Labor-intensive and expensive; (2) safety issues (biosafety level 2 laboratory needed); (3) can cause immune/inflammatory responses *in vivo*; (4) no site-specific integration into the genome with recombinant vectors; risk of insertional mutations	Low	Onset ~2 weeks after transduction; high levels of expression	~5 kb	Yes
Lentiviral vectors	Cell types: Cell lines and primary neuronal cells, including mature, fully differentiated neurons, *in vitro*; whole nervous system, including adult nervous system, *in vivo* Applications: (1) Efficient *in vitro* (cell lines, primary postmitotic neurons) and *in vivo* gene delivery; (2) transient and stable transduction and inducible expression; (3) transduction of neurons in brain slices; (4) suited for quantitative and biochemical analyses because of very high transduction efficiencies	(1) Very high transduction efficiencies in dividing and nondividing mammalian cells (2) easy to produce high-titer stocks and simple transduction procedure; no/little purification of viruses needed; (3) low cell toxicity; (4) integration into the genome; (5) transduction of specific cell types, possible via pseudotyping of viral vectors	(1) Labor-intensive and expensive; (2) safety issues (biosafety level 2 laboratory needed); (3) no site specific integration into the genome; possibility of insertional mutagenesis	Low	Few hours after transduction; high levels of expression	~10 kb	Yes
Herpes simplex viruses	Cell types: Cell lines and primary neuronal cells, including mature, fully differentiated neurons, *in vitro*; whole nervous system, including adult nervous system, *in vivo* Applications: (1) Efficient *in vitro* (cell lines, postmitotic primary neurons) and *in vivo* gene delivery; (2) tracing of neuronal pathways *in vivo*; (3) transduction of neurons in brain slices	(1) Natural neurotropism; (2) very high transduction efficiencies in dividing and nondividing mammalian cells; (3) no risk of insertional mutagenesis, as there is no genome integration; (4) large insert size allows transduction of multiple genes or genomic regions	(1) Labor-intensive and expensive; (2) safety issues (biosafety level 2 laboratory needed); (3) immune/inflammatory responses *in vivo*	High; lower with amplicon vectors	Few hours after transduction; high levels of expression, decreases within the first few weeks	>100 kb possible (with amplicon vectors)	No

(*Table continues.*)

Table 1. Continued

	Best suited for	Strengths	Limitations	Toxicity	Onset, level, and duration of expression[a]	Maximum insert size	Genome integration
Microinjection	Cell types: Large and robust neurons (neuronal cell lines; differentiating and mature primary neurons) *in vitro* Applications: (1) Analyses requiring low numbers of transfected cells where specific cells are targeted, e.g. live imaging of individual neurons *in vitro*; (2) introduction of molecules other than nucleic acids; (3) injection into a specific subcellular region/compartment	(1) Possible to inject substances that cannot be synthesized by a cell, e.g. labeled RNAs, neutralizing antibodies; (2) transfection of specific cells or cell types in a mixed cell culture; (3) possibility to inject into the nucleus (e.g. normal nuclear processing of RNAs)	(1) Low transfection rates, limited by the features of the cell type (larger and more robust neurons are easier to inject and have a higher chance of surviving); (2) very time consuming; (3) relatively expensive equipment	Poor survival rate because of physical damage of neurons during injection	Expression plasmids: typically within hours; fluorescent signal of injected labeled RNAs very low	No limit	No
Biolistics	Cell types: All cell types in entire brains (*in vivo*) and tissue slices; cultured cells *in vitro*, including neuronal cell lines as well as differentiating and mature primary neurons (not suited for early differentiation stages however, as cells must be firmly adherent to substrate so as to not detach after bombardment with gold particles) Applications: (1) Experiments on individual neurons (including mature neurons) in entire brains and spinal cords; (2) relatively high transfection rates *in vivo* without the need for special safety measures; (3) combined with two-photon microscopy: imaging of cells deeper inside the tissue; (4) electrophysiological recordings on individual cells	(1) Analyses on individual neurons in normal cellular context; (2) quick protocol; (3) neurons deep into a tissue can be transfected	(1) Relatively expensive equipment and reagents; (2) Relatively low transfection efficiencies (typically ~2%); however, recently improved protocols lead to higher transfection efficiencies of up to 10% (cultured neurons) and up to 34% (slice cultures); (3) collateral tissue damage *in vivo*	Significant cell damage caused by high pressure and accelerated gold particles; recently developed hand-held gene gun or use of "mash" show significant improvement	Typically within 1–2 d after bombardment; near-physiological expression generally persists for a minimum of 3–4 d in cell culture, up to 7 d in slices	No limit	No

[a]In addition to the parameters specific to each method, as described in this table, the onset, level, and duration of expression varies, e.g., with the plasmid and promoter used and the expressed construct, as well as the DNA concentration used.

consistently high rates for a range of such plasmids in primary rat (E17) neuronal cells (Zeitelhofer et al., 2009a).

Recently, neural progenitor cells (NPCs) have been transfected via nucleofection with rates of up to 50–60% (~80% stably transfected cells after antibiotic selection) without compromising their proliferation or differentiation potential (Dieterlen et al., 2009). This is particularly interesting because the differentiation of NPCs into different neural cell types bears the promise of repairing or regenerating the nervous system, and the ability to introduce genetic material may thus have important therapeutic implications. While neurons from postnatal and adult brains can also be electroporated or nucleofected (Knoll et al., 2006), these techniques tend to be more effective with younger neuronal cells.

Another recent modification of electroporation, single-cell electroporation, allows the transfer of expression plasmids into individual cells *in vivo* (Kitamura et al., 2008). To achieve this, a target neuron—up to 1 mm deep into the brain tissue—is identified and visualized in the intact brain by two-photon microscopy. The neuron is subsequently electroporated with a high resistance patch pipette containing the plasmid DNA. Following the electroporation, transfected neurons can be imaged and/or targeted for whole-cell patch-clamp recordings. Importantly, such neurons were healthy and displayed normal electrophysiological properties, and stable transgene expression could be observed even months after the electroporation (Judkewitz et al., 2009). Target cells can also be electroporated with different constructs at subsequent time points. This allows the expression of multiple transgenes inside the same cell, which is useful when assessing temporal effects of gene expression during neural differentiation and patterning *in vivo*. Crucially, this method allows analyses of the functional integration of individual neurons within a network. Single-cell electroporation can thus be used to study the role of genes and individual cells in neural circuits, e.g., their activity, plasticity, and behavioral characteristics.

Chemical transfection methods

Ca^{2+}-phosphate/DNA coprecipitation

The Ca^{2+}-phosphate/DNA coprecipitation method is one of the best established transfection methods and very commonly used to transfect different types of primary neuronal cells as well as cell lines *in vitro* (Dahm et al., 2008). It is cost effective, does not require specialized equipment, and very easy to establish. This method can be used to transfect neurons at all stages of differentiation, including those that have already formed a functional neuronal network. The basic principle involves the formation of DNA crystals with the Ca^{2+} ions in the phosphate buffer that then precipitate onto the cells and are presumably taken up via endocytosis. In proliferating cells, the DNA can subsequently enter the nucleus when the nuclear envelope breaks down during mitosis. In postmitotic cells such as neurons, entry into the nucleus is more difficult and the expression rate consequently reduced. Therefore, the transfection efficiency generally lies between 1 and 5% and, even after optimization, rarely reaches 30% (Goetze et al., 2004).

An advantage of the Ca^{2+}-phosphate/DNA coprecipitation is that the time course and level of protein expression can easily be varied by titrating the DNA concentration via alteration of the amount of

plasmid used (Dahm et al., 2008). This is an advantage, as rapid and strong expression reduces the period in which the overexpressed protein occurs in (near) physiological levels before potentially leading to overexpression artifacts. Importantly, when optimized, transfection via Ca^{2+}-phosphate/DNA coprecipitation results in good cell viability. These advantages make the Ca^{2+}-phosphate/DNA coprecipitation ideally suited for applications requiring low numbers of transfected cells that show physiologically normal behavior. These include, for example, live imaging experiments focusing on single cells *in vitro* found within a neuronal network in culture (low numbers of transfected cells in complex neuronal networks are an advantage when dendrites and axons of individual neurons have to be identified) or the evaluation of neuronal phenotypes after RNAi (Dahm et al., 2008). This method can also be applied to study the subcellular localization of proteins and the colocalization of proteins and RNAs in developing and mature neurons.

Lipofection

Conventional lipid-mediated gene delivery is based on cationic lipid molecules. These form small unilamellar liposomes that interact with negatively charged nucleic acids (NAs) and facilitate the fusion of the lipid:NA complex with the negatively charged plasma membrane. Cationic lipid molecules are often combined with a neutral helper lipid, which mediates the fusion of the liposome with the membrane. Newer generations of lipofection reagents, however, use nonliposomal lipids to form a complex with the NAs. This complex is believed to be endocytosed and released into the cytosol. Nonliposomal lipids have been demonstrated to have high transfection efficiencies in a wide variety of cell types, including primary neuronal cells, such as cerebellar granule neurons (Butcher et al., 2009). Importantly, they often work in the presence of serum, which generally results in improved cell growth and viability and may reduce the cytotoxic effects of the transfection.

Lipofections are technically simple, require no specialized equipment, show high reproducibility and low toxicity, and generally require little optimization (although several reagents may have to be tested to achieve the best results with unconventional cell lines/types). They are suitable for both transient and stable transfections of a variety of cell lines. Transfection efficiencies are usually very high when used with cell lines (up to 85%), but can vary considerably between cell types. However, the same lipids, when used to transfect postmitotic neurons, tend to give poorer results (typically 1–5%), although maximum values of up to 30% have been reported for primary neurons (Dalby et al., 2004). These comparatively low transfection efficiencies, while affording near endogenous expression levels (Washbourne and McAllister, 2002), limit the application of lipofection for plasmid-based vectors in postmitotic cells.

For RNAi, cytoplasmic delivery of small interfering RNAs (siRNAs) is sufficient. Lipofection reagents efficiently transfer siRNAs, microRNAs, or other oligonucleotides into postmitotic neurons (with up to 83% efficiency in primary rat hippocampal neurons) (Tonges et al., 2006). Finally, there is great interest in lipid-based DNA delivery for gene therapy, as this method has a lower risk of causing mutations and immune responses than virus-based delivery (Zhdanov et al., 2002).

Virus-based transfection methods

Viral vectors have received much attention recently and have become powerful tools for gene delivery *in vitro* and *in vivo*. In cultured cells, viruses are primarily used to achieve stable genomic integration and an inducible expression of transgenes. *In vivo*, viruses are often the only viable option when aiming at efficiently introducing transgenes into specific cell types, as is needed, for instance, in gene therapy. Importantly, the viruses described here can infect postmitotic mature (adult) neurons *in vitro* and *in vivo*.

Another substantial advantage of viral gene transfer (transduction), both *in vitro* and *in vivo*, is the extremely high efficiency. This is not surprising, since viruses evolved to infect cells and express their genetic material. A second major advantage is that different viruses have distinct tropisms. This can help restricting transgene expression to a subset of cell types, greatly facilitating *in vivo* studies. Given the diversity of biological characteristics of different viruses (tropism, genome integration, strength, duration of expression, etc.), the choice of viral vector depends on the gene of interest (GOI), the targeted cell type, and the experimental application.

Despite these advantages, viral vectors have important limitations. Although most recombinant viral vectors in use today are replication incompetent and thus comparatively safe to use, they still require biosafety level 2 facilities. Furthermore, despite the commercial availability of complete kits, transduction protocols require preparations of recombinant vectors in packaging cell lines and a subsequent purification of virus particles. Packaging cells express viral gene products necessary for the production of infection-competent virions. While this step is time consuming, it ensures that the modified virus used cannot replicate in the target cells after transduction. In addition, some viruses [adeno-associated viruses (AAVs), modified herpes simplex viruses (HSVs)] require the coinfection of packaging cells with a wild-type helper virus to produce infectious virions. This results in a contamination of the supernatant (from which the infectious virions are purified) with helper viruses, which often cannot be fully eliminated during the preparation of the viral stock (Epstein, 2009) and can have cytotoxic effects (White et al., 2002).

Adenoviruses

Adenoviruses (AdVs) infect target cells, including postmitotic neurons, with high efficiency and in multiple copies. The first generation of adenoviral vectors is cytotoxic if used at high titers and shows late onset and low levels of expression (Washbourne and McAllister, 2002). Moreover, these vectors (as adeno-associated vectors) can cause significant immune responses when used *in vivo* (Lowenstein et al., 2007; Buning et al., 2008). Recently, a new generation of adenoviral vectors has been designed to overcome this limitation. The genomes of high-capacity, helper-dependent adenoviruses do not encode any viral proteins and, as a consequence, do not elicit immune responses (Lowenstein et al., 2007).

AdVs do not integrate into the host genome and are therefore suitable for transient expression of GOIs. Since the expression can persist for weeks to months, recombinant AdVs are also often used to generate inducible expression systems *in vitro* and *in vivo*. A drawback of AdVs when targeting neurons is their preferential infection of glial cells, which can limit the transductions of slices or tissues.

Adeno-associated viruses

AAVs have emerged as very powerful tools for gene delivery into neurons. Distinct capsid proteins expressed by different AAV serotypes result in the use of different cell surface receptors for cell entry and thus specific tropisms (Buning et al., 2008). Several of these AAV serotypes have been demonstrated to infect primary neurons with high efficiency and low toxicity (Royo et al., 2008), with AAV-2 being the most commonly used. Wild-type AAVs stably integrate into the human genome in a

site-specific manner. Recombinant AAV-based vectors, however, integrate rarely and randomly because they lack the viral *rep* gene (Buning et al., 2008).

Since AAVs are naturally replication incompetent, they require a coinfection with an unrelated, wild-type helper virus (e.g., AdV, HSV) to supply essential gene products for the production of infectious virions. The new generation of recombinant AAVs is "helper-free" (while remaining replication deficient), eliminating the handling of an infectious, wild-type helper virus and simplifying the procedure. Moreover, by removing wild-type virus from the gene delivery procedure, the immune response of target cells is minimized. Limitations of AAVs are the late onset of transgene expression (~2 weeks after infection), which hampers experiments with limited time frames, and a maximum insert size of ~5 kb, restricting their use to smaller transgenes (Washbourne and McAllister, 2002).

Lentiviruses

In contrast to other retroviruses, lentiviruses [including human immunodeficiency virus (HIV)] are capable of infecting nondividing cells. They insert into the host genome and are thus best suited to generate stable transgenic cell lines. Together with their high transduction efficiencies and low toxicity, this makes lentiviral vectors very useful for the generation of inducible expression or knock-down systems *in vitro* and *in vivo*. Since stable integration into the host genome carries the risk of insertional mutations, however, recent developments of nonintegrating lentiviral vectors are promising, especially for *in vivo* applications (Rahim et al., 2009).

To broaden the potential uses of lentiviruses, recombinant HIV-1 vectors were pseudotyped, i.e., HIV-1 envelope proteins, which naturally recognize CD4 receptors on their target cells, were substituted by proteins from other viruses to alter the tropism of the virions (Cockrell and Kafri, 2007, and references therein). This allows the targeting of a wider spectrum of specific cell types with high efficiencies.

Rrecent developments have also reduced the risk posed by replication-competent lentiviruses as follows: (1) viral packaging elements are provided on individual plasmids that need to be cotransfected into packaging cells to produce virions; and (2) six of HIV-1's nine genes encoding important virulence factors have been eliminated without affecting its gene-transfer ability. Most of the commercially available lentiviral systems are based on these third generation vectors, providing a relatively safe and efficient way for transient or stable expression of GOIs or RNAi in dividing and nondividing cells.

Herpes simplex viruses

HSV-1 was the first virus used for gene delivery into neurons. HSVs are particularly attractive for neuroscience, as they naturally infect neurons with high efficiency and can carry large inserts. Furthermore, the ability of HSVs to be transported and transferred across synapses in a retrograde fashion can be used to trace neuronal pathways (Simonato et al., 2000).

Recombinant HSV-1 and amplicon (plasmid)-based vectors have been developed (Epstein, 2009). Amplicon vectors carry almost no genes of the HSV-1 genome (and are thus nontoxic), but have a transgene capacity of up to 150 kb. This allows for the insertion of multiple copies of a transgene or of large genomic regions, including regulatory elements (Epstein, 2009). However, they require a wild-type helper virus (HSV-1) for replication and packaging. A major drawback is the difficulty of producing high-titer stocks of vector particles free of helper virus (Epstein, 2009), which can lead to cytotoxic effects and/or immune responses. The recent development of helper virus-free systems is therefore promising (Fraefel, 2002).

Despite their widespread preclinical use, vector toxicity remains a concern when working in patients. Another limitation, especially for long-term *in vivo* approaches, is the reduced recombinant gene expression within a few weeks after gene transfer. Recently specific proteins in the HSV-1 virus particle have been associated with the shut-off of transgene expression (Liu et al., 2009), suggesting ways to improve expression over longer periods of time.

Physical transfection methods

Microinjection

During microinjection, nucleic acids are injected into the cytoplasm or nucleus of cells with fine glass capillaries. While microinjection has been used with mammalian neurons, it is more frequently used in experiments with (larger and more robust) invertebrate neurons. A major disadvantage of this technique is the substantial stress caused by disrupting the plasma membrane during microinjection, which results in very low survival rates for many types of neurons. Importantly, to ensure that the injection did not compromise neuronal integrity, function, and/or subsequent development, appropriate controls have to be included in every microinjection experiment (Zhang and Yu, 2008). Despite these drawbacks, this technique has a substantial advantage: it allows the injection of substances that cannot be synthesized by the cell. For instance, directly labeled RNAs (including microRNAs) can be used to follow their subcellular localization and turnover or their association with specific proteins or other RNAs (Schratt et al., 2006). The injection of molecular beacons, which emit a fluorescence signal only upon binding to their target RNA, is an alternative approach to analyzing gene expression or RNA localization in living cells. In addition to injecting RNAs, microinjection can be used to assess the effect of neutralizing antibodies or toxins. Microinjection also allows targeting defined cells in a mixed cell culture or neuronal network. Finally, unlike with other transfection methods, exogenous material can be injected into specific subcellular regions or compartments.

Biolistics

Biological ballistics, or biolistics, is based on the injection of DNA-coated gold particles by using a motive force, such as high helium pressure (Lo et al., 1994). It can be applied to transfect cells in cultures, tissue slices, or living organs, thus allowing experiments on individual neurons in the context of an entire brain or spinal cord region. This is of particular importance when trying to assess the influence of the neuronal and glial microenvironment and the three-dimensional integration of a neuron within its natural cellular context, which cannot be mimicked in cultures of dissociated neurons or neuronal cell lines. Neurons transfected by this technique can be imaged and targeted for whole-cell patch-clamp recordings. Moreover, the environmental conditions of the slices can be manipulated following the transfection to simulate pathological conditions, such as hypoxia, to mimic stroke or assess the effects of drug treatments.

Compared with viral transfections, biolistic gene transfer is considerably faster, simpler, and does not require additional safety measures. Similar to electroporation, biolistics provides higher transfection rates than lipofection in slice cultures. Importantly, constructs can be transferred to depths of up to 100 μm into a tissue or organ (Murphy and Messer, 2001). The efficiency of biolistics (transfection rate and penetration depth), however, has to be counterbalanced with the damage to cells and tissues caused by the particles (size/degree of particle agglomeration). Finally, biolistics also allows the targeting and *in vivo* analysis of cell types for which no transgenic

animals are available, e.g., because of the lack of cell type-specific promoters.

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