Published in final edited form as:

Neuromethods. 2011 ; 56: 185–222. doi:10.1007/978-1-61779-077-5_9.

Introducing cloned genes into cultured neurons providing novel *in vitro* models for neuropathology and neurotoxicity studies

Marcelo Farina¹, Jordi Berenguer², Sebastián Pons², João Batista Teixeira da Rocha³, Michael Aschner⁴

¹Departamento de Bioquímica, CCB, Universidade Federal de Santa Catarina, Florianópolis, Santa Catarina, Brazil

²Department of Cell Death and Proliferation, IIBB, CSIC-IDIBAPS, Barcelona, Spain

³Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria - RS, Brazil

⁴Departments of Pediatrics and Pharmacology, and the Kennedy Center for Research on Human Development, Vanderbilt University Medical Center, Nashville, TN, USA

1. Introduction

The introduction of nucleic acids into cultured cells has been widely used to study the gene-specific function. With the advent of novel techniques for molecular cloning and cultured cell transfection, innovative approaches have provided relatively rapid and efficient ways to elucidate the effects of genes (and their encoded proteins) in the living cells. With particular emphasis on cultured neuronal cells, the transfection of nucleic acids can be used to overexpress genes that encode proteins involved in neurodegenerative and neuroprotective events, as well as in neurotransmission, antioxidant defenses, energetic metabolism and several others physiological phenomena related to the neuronal homeostasis.

Many books have been written on manipulating gene expression in cultured cells derived from different sources. Given space limitations, this chapter cannot treat the subject in depth; rather, it discusses the fundamentals of dealing with vectors (especially plasmids), bacterial transformation, plasmid maximization and purification, and transfection of cultured neuronal cells. For additional topics and details on protocols concerning molecular cloning and cultured cell transfection, the reader is referred to an excellent text listed at the end of the chapter (Recommended Reading).

2. Cloning techniques

Molecular cloning techniques describe how to isolate a DNA fragment (i.e. a complete gene, part of it or a promoter), modify it and insert it into another DNA molecule. This receiver DNA molecule is generally called vector, which allows producing high amounts when

Correspondence should be addressed to: Marcelo Farina, Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, 88040-900, SC, Brazil, farina@ccb.ufsc.br; or Jordi Berenger, Department of Cell Death and Proliferation, IIBB, CSIC-IDIBAPS, Rosselló, 161 6^a and 7^a floor - 08036, Barcelona, Spain, jbfngi@iibb.csic.es.

transfected into bacteria (cloning vectors). Some vectors may also use the transcriptional machinery of a eukaryotic cell, like a neuron, and can be used to study the function of the inserted DNA sequence. Due to their many different applications in the Neuroscience field, in this section, we will focus on their generation and handling.

2.1. Vectors

Vectors are DNA molecules derived from natural extrachromosomal DNA molecules found in bacteria and yeast (plasmids, which are double-stranded generally circular DNA) or from bacteriophages and other viruses. Since the 1970s, thousands of vectors have been created by cutting and joining sequences from different sources (other vectors, prokaryotic or eukaryotic sequences) in an attempt to provide them useful features. Some of these features are common between the majority of vectors, such as (i) the replication origin (ori), required to be propagated into the host cell, (ii) an antibiotic resistance to eliminate bacteria that have not been transformed [commonly Ampicillin resistance (Amp^r), Kanamycin resistance (Km^r) or Cloramphenicol resistance (Cm^r)] and (iii) a multiple cloning site (MCS), used to open the circular molecule and insert the sequence of interest. Other features will depend on the particular application of the vector.

2.1.1. Overexpression vectors—Overexpression vectors are designed to produce high quantities of a protein encoded by the cloned sequence. Of particular importance, the overexpression of the specific protein allows for predicting its role in neurotoxic/neuroprotective events (1). Figure 1 shows an example of the overexpression vector (pCIG). These vectors need to have a strong promoter in an upstream position to the multiple cloning site (MCS), as well as a polyadenylation signal at a downstream position, which must be recognized by the transcriptional machinery of the transfected eukaryotic cell. Common promoters used for overexpression in eukaryotic cells are the human Cytomegalovirus immediate-early (CMV) promoter/enhancer (2–4) and the Simian Virus 40 early promoter/enhancer (5). It is important to note that the activity of a promoter depends on the transfected cell type (6). According to our experience, the Chicken β -actin promoter, in combination with the CMV immediate-early promoter-enhancer, represent a good choice when working with cultured neuronal cells. Particularly, the vector depicted in Figure 1 presents a specific region that confers ampicillin resistance (Amp^r).

As discussed later, the process by which a vector enters the eukaryotic cell (transfection) never reaches a complete efficiency. Accordingly, after a transfection, a mixture of transfected and non-transfected cells is obtained. Overexpression vectors often have specific features allowing for distinction between transfected and non-transfected cells. One of them is the use of reporter genes, such as Green Fluorescent Protein (GFP) or its derivatives. GFP is a protein cloned from the jellyfish *Aequorea victoria* that emits green fluorescent light when exposed to blue or ultraviolet light. Interestingly, GFP does not alter normal cellular homeostasis. Consequently, its use as well as similar vectors allow for distinction between transfected and non-transfected neurons by irradiating them with blue light in a fluorescence microscope. Another way to identify transfected cells is the fusion of the cloned sequence to an epitope-Tag that is inserted in specific commercial vectors. Epitope-tag sequences (like

myc-Tag or Flag-Tag) encode epitopes easily identifiable with the commercially available antibodies.

Overexpression vectors can also be used to decrease the function of a specific protein. In fact, it is possible to mutate a gene and convert it into a dominant-negative form that, when overexpressed, blocks the function of the wild-type gene (7, 8). This strategy takes advantage of the fact that the mutated overexpressing protein can compete for specific substrates of the non-mutated protein.

2.1.2. Luciferase vectors—Luciferase vectors are designed to clone regulatory regions of the genomic DNA (*i.e.*, promoters, enhancers or transcription factor binding sites) to evaluate their function from a quantitative point of view. In these vectors, the MCS is located upstream a sequence encoding for luciferase protein, driving its expression. Luciferase, which is a protein derived from the firefly *Photinus pyralis*, catalyzes the reaction in which luciferin is oxidized to oxyluciferin and light (9, 10), usually in the green to yellow region, typically 550–570 nm. Thus, the relative activity of a regulatory sequence cloned into a luciferase vector and transfected into cells can be measured through the luciferase reaction (light emission) (11, 12).

2.1.3. Other purpose vectors—There are four different types of vectors (plasmids, bacteriophages and other viruses, cosmids and artificial chromosomes) with several properties that make them useful in molecular biology. Of particular importance to neuroscience studies, inducible promoter vectors can start transcription only in response to a concrete signal (*i.e.*, an antibiotic or a naturally secreted molecule) and Short Interfering RNA (siRNA) vectors specifically down-regulate the expression of a particular gene. Since the aim of this section is to provide a brief, simple, but comprehensible guide for gene manipulation and transfection into cultured neuronal cells, we describe only two of the most common vectors used in molecular biology. For more information about vectors in general, please check the Recommended Reading.

2.1.4. Plasmid isolation protocol—There are several methods for the isolation of plasmid DNA from bacteria. Many of them are based on alkaline lysis with Sodium Dodecyl Sulphate (SDS). They are very fast, simple and provide good yield. The purification is performed in three steps. In the first step, bacteria are resuspended in a buffer containing EDTA. EDTA destabilizes the cell wall by chelation of divalent cations and inhibits DNases. In the second step, a buffer at high pH containing NaOH and SDS is added. In this step, SDS breaks cell walls, disrupts cell membranes and proteins and chromosomal DNA are denatured while plasmid DNA is released into the supernatant. Base pairs of chromosomal and plasmid DNA are disrupted, but plasmid DNA remains topologically constricted. In the third step, -OH ions are replaced by potassium ions. This step renaturates plasmid DNA while precipitating chromosomal DNA. This protocol gives DNA of sufficient quality for many molecular cloning techniques, but purification steps can be added if needed (*i.e.* phenol:chlorophorm extraction, see Recommended Reading).

1. Pick a single colony of plasmid-transformed bacteria into 3 mL Luria-Bertani (LB) medium (10% bacterial peptone, 5% yeast extract and 10% NaCl). Grow it

overnight or until log phase is achieved (do not let it grow too long: if the antibiotic is fully processed by bacteria, the plasmid will be lost).

2. Transfer 1.5 mL of the culture to propylene tube and centrifuge at $13,000 \times g$ for 20 min. Store the rest of the culture at 4°C and pour off the supernatant.
3. Add 100 µL of buffer P1 (Resuspension buffer: 50 mM Tris-HCl - pH 8.0, 10 mM EDTA, 100 µg/mL RNase A). Resuspend the pellet vigorously.
4. Add 100 µL of P2 buffer (Lysis buffer: 200 mM NaOH, 1% SDS). Mix by inversion 10 times.
5. Add 100 µL of P3 buffer (potassium acetate 3M - pH 5.5). Mix by inversion 10 times. Centrifuge at $13,000 \times g$ for 5 min. Harvest supernatant.
6. Add to the supernatant 2 volumes (600 µL) of cold 100% ethanol. Mix by inversion 10 times. Centrifuge at $13,000 \times g$ for 5 min.
7. Pour off the supernatant and wash the pellet with 1 mL of 80% ethanol. Centrifuge at $13,000 \times g$ for 5 min. Pour off the supernatant and let the pellet dry.
8. Resuspend the pellet in 30 µL of water (stable at -20 °C for several weeks).

2.2. Manipulating genes: cutting, pasting and inserting into bacteria

Recombinant DNA technology arose in the early of 1970s as a result of great advances in the field of the bacterial and bacteriophage's biology (13). These advances led scientists to isolate enzymes able to modify DNA: restriction endonucleases (RE), which cut DNA in concrete positions, or DNA ligases, which join DNA molecules. The insertion of these DNA molecules into bacteria (transformation) allowed the production of large amounts of the inserted gene.

2.2.1. Restriction Endonucleases—Restriction Endonucleases (or restriction enzymes; RE) are bacterial proteins involved in the recognition and elimination of foreign DNA sequences inside the cell, like bacteriophage's DNA (14–17). They recognize specific nucleotide sequences that are cleaved when unmethylated. To avoid cleavage of the own DNA, bacterial RE can methylate DNA to “label” it as its own molecule, protecting against cleavage (18–21).

There are at least three types of RE, based on their composition, the point of recognition, methylation and cleavage, and cofactor requirements (22):

Type I REs are composed of a single enzyme with different subunits for recognition, methylation and cleavage. These enzymes cut at a site that differs, and is at some distance (at least 1000 bp) away from their recognition site. They need several cofactors for catalytic activity, such as adenosine triphosphate (ATP), s-Adenosyl methionine (AdoMet) and Mg^{2+} .

Type II REs are composed of two different enzymes with one subunit each, which recognize the same target site, usually a palindromic 4–8 bp long sequence. One of them methylates the sequence and the other one cuts it. Usually, the only cofactor required for their activity is Mg^{2+} , with no need for ATP or AdoMet. These facts (possibility to separate cleavage and

modification activities, recognition and cleavage at the same specific sequence and requirement of only Mg^{2+} as a cofactor) make them very useful for molecular cloning. In the last several decades, dozens of type II REs have been isolated and several companies have commercialized them as useful tools to perform restriction reactions. Typically, a restriction reaction contains only the target DNA, a specific buffer and the restriction enzyme. Furthermore, REs often have similar buffer requirements, making possible to cleavage the target DNA at two sites in only one step reaction.

Type II REs can make two kinds of cleavage in the double strand (ds) DNA. Since the target sequence is generally palindromic, that is, both strands have the same sequence when read from 5' to 3', the RE makes a symmetric cleavage. In the case of EcoRI cleavage, the enzyme recognizes the following sequence,

and cuts between the G and the first A. Then, after the cleavage, the new DNA ends generated will be,

which are cohesive or “sticky” ends, due to the 5' “overhang”. These regions can anneal to other DNA fragments cut by the same RE. Alternatively, some REs cut dsDNA at the middle of the recognition site. This is observed for EcoRV, whose recognition site is

In this case, no “overhang” is created in the cleavage site and the created ends are called “blunt” ends.

Type III REs are composed by one enzyme with two different subunits: one that recognizes and methylates the same sequence and another that cleaves at 20–30 bp away from their recognition site. They need ATP and AdoMet for catalytic activity.

2.2.1.1. Restriction protocol: Here, we describe a simple protocol to cut DNA using the RE EcoRI provided in the Fermentas® Five Buffer System. Where other REs are used, the buffer and temperature might differ. Other companies have similar systems.

1. In a clean tube, mix the target DNA (between 0,1 to 2 µg), 2 µL of 10 × EcoRI buffer (final concentration: 50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM MgCl₂, 100 mM NaCl, 0,02% Triton X-100, 0.1 mg/ml BSA), 0.5 µL EcoRI (5 units) and water to a final volume of 20 µL.
2. Incubate at 37°C/1h.
3. Add 4 µl of 6X DNA loading buffer (0,09% Bromophenol Blue, 60% Glycerol, 60 mM EDTA) and run the sample in agarose gel with DNA staining dye (*i.e.*, Ethidium bromide or Sybr-Green) and a commercial DNA ladder (*i.e.*, 1Kb plus DNA ladder from Invitrogen, Carlsbad, CA) to separate RE and verify the restriction. The concentration of agarose will depend on the fragments size. Typically, use 1.5–2% for fragments smaller than 1 kb and 0.5–1% for fragments larger than 1 kb.

2.2.2. DNA ligases—DNA ligases are enzymes that close nicks in the phosphodiester backbone of DNA. They are found in eukaryotes, prokaryotes and viruses, and are involved in important cellular processes, such as the sealing of Okazaki fragments during replication (23). Prokaryotic DNA ligases use NAD⁺ as a cofactor while eukaryotic and viral DNA ligases (like T4 bacteriophage DNA ligase, the most used in recombinant DNA technology) use ATP (24, 25). The reaction catalyzed by DNA ligases occurs in two steps, where ATP is crucial for the formation of an AMP-DNA complex (Figure 2).

When two DNA molecules are cut with the same RE, the sticky ends of the double chain can anneal by base-complementarities. Since the four generated nicks are substrates for DNA ligase, a new artificially recombinant DNA molecule can be generated after ligation (*i.e.*, the overexpression vector with a human gene cloned in its MCS - Figure 3A).

During the ligation reaction, another possibility is the closing of the pattern DNA molecule (Figure 3A) with no addition of the insert (recircularization). This event is particularly undesired when working with vectors, because the empty recircularized vector can be transformed and propagated into bacteria. Since recircularization of a vector is usually more frequent than insert-vector ligation, it is difficult to discriminate bacteria with the empty vector from those containing the insert-vector. There are some strategies to minimize this problem. Ligation reactions are usually performed at high DNA concentration and high insert:vector ratios (typically 4:1 to 6:1) to maximize intermolecular reactions rather than intramolecular ones. Another strategy to minimize the occurrence of recircularization is to cut both ends of the vector and insert with two different, incompatible RE (Figure 3B). Consequently, one end of the molecule cannot hybridize with the other one, avoiding recircularization. When this approach is not possible, a vector dephosphorylation step is included in the cloning protocol, between restriction and ligation (Figure 3C). Alkaline phosphatases, such as calf or shrimp alkaline phosphatases, are able to dephosphorylate 5' phosphate ends of DNA. When a vector is dephosphorylated, it can no longer recircularize

because DNA ligase needs 5'-phosphate residue to catalyze ligation reaction. This way, the only possible ligation reaction is between 5' phosphate group of the insert and 3'-OH group of the vector, leaving a nick in each chain, which can be repaired in subsequent replications of the plasmid.

2.2.2.1 Ligation protocol: In this section, we describe a protocol for ligation of a cohesive double-restricted insert into a vector with the Fermentas® system. Other companies have very similar protocols. For blunt-end ligation, see Recommended Reading.

1. Run a small sample of the vector and the insert in an agarose gel with DNA loading buffer (see restriction protocol, item 2.2.1.1).
2. Quantify the relative intensity of bands [visually or with the help of software (*i.e.*, Bio-Rad's Quantity-One)].
3. Transform the relative intensity of the bands to relative quantity of DNA. Intensity will depend only on the amount of DNA and the size of the molecule (a band of 2kb has double intensity than a band of 1kb, if they are present in similar amounts).
4. In a clean tube, mix the vector and insert at a 1:4–1:6 ratio (if the amount of DNA is known, around 25–50 ng of vector are recommended). Add 2 µL of 10 × T4 DNA ligase buffer (final concentration: 40 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP), 0.5 µL T4-DNA ligase and water to a final volume of 20 µL. Repeat the ligation in another tube replacing the insert with water (negative control). Negative control-transformed cells will provide an estimate of the proportion of recircularized vector-transformed cells.
5. Incubate the reaction: the optimum temperature for DNA ligase is 37°C, but annealing of sticky ends is compromised at this temperature. Usually, 1 h at room temperature works best. For harder ligations, incubate overnight at 17°C.
6. Store at –20°C or proceed with bacterial transformation.

2.2.3. Competence and transformation—The process by which bacteria remove free DNA from the extracellular environment is called transformation and the ability to be transformed is called competence. Bacteria naturally acquire competence and are transformed to transfer genetic information (such as antibiotic resistances) between individuals. This phenomenon was discovered by Frederick Griffith (26), but the techniques to make competent and transform *E. coli in vitro* were developed only in the 1970s (27, 28). In molecular cloning, *E. coli* can be transformed by chemical transformation, electroporation, biolistic methods, or sonic transformation. In this section we describe a simple chemical method to make competent cells and transform them.

2.2.3.1. Preparation of competent cells and transformation protocol: This protocol is based on the ability of polyethylene glycol (PEG) to induce bacterial transformation (29, 30). It is very simple and provides good transformation efficiency.

2.2.3.1.1. Preparation of competent cells

1. Grow 5 mL of cells in Luria-Bertani (LB) medium (10% bacterial peptone, 5% yeast extract and 10% NaCl) without antibiotic, overnight, at 37°C (250 rpm).
2. Inoculate 1 mL of the culture into 250 mL of LB until the optic density at 600 nm reaches 0.6.
3. Pellet the cells by centrifugation at $1000 \times g$ for 10 min. Pour off the supernatant and let the tube over paper to dry excess of medium.
4. Resuspend the cells in 25 mL filter-sterilized (0.22 μ m) TSB (LB pH 6.1, 10% PEG (3350 Da), 5% DMSO, 10 mM MgCl₂, 10 mM MgSO₄).
5. Let on ice during 10 min and aliquot in 0.5 pre-chilled tubes. Freeze in liquid N₂ or ethanol/dry ice bath. Store at -70°C.

2.2.3.1.2. Transformation

1. Thaw one aliquot of competent cells on ice.
2. Mix 20 μ L KCM (KCl 0.5 M, CaCl₂ 0.15 M, MgCl₂ 0.25 M) with the ligation mixture (derived from protocol 2.2.2.1) and add water to 100 μ L.
3. Pipette 100 μ L of competent cells to each tube of DNA/KCM and mix gently and leave it for 20 min on ice.
4. Leave 10 min at room temperature and add 800 μ L of LB medium (without antibiotic).
5. Incubate for 1 h at 37°C under agitation
6. Centrifuge at $14000 \times g$ during 15 minutes.
7. Remove 850 μ L of LB, resuspend the cells and plate them in LB-Agar (LB with 15% Bacteriological Agar) with antibiotic.

2.3. DNA amplification and site-directed mutation by Polymerase Chain Reaction (PCR)

Molecular cloning techniques had important limitations until the second half of 1980s. The discovery of RE and ligases allowed for novel methods for cloning DNA sequences into plasmids but, until the description and improvement of PCR, the only way was to construct DNA libraries. A DNA library is constructed by cutting (mechanically or by restriction) genomic DNA (gDNA) or complementary DNA (cDNA, which is DNA synthesized in vitro by reverse transcription) into thousands of unknown fragments that can be cloned into phagemid vectors. Once the library is constructed, it is necessary to identify the clones of interest by laborious screening strategies based on nucleic acid probe hybridization, functional studies and immunological recognition of products by antibodies. The use of these techniques presented several difficulties and limitations. One of them was the fact that the fragments were randomly generated and then were frequently lost, incomplete or contained undesired regulatory regions. On the other hand, sometimes there were no screening strategies to find the sequence of interest. PCR technique abolished these problems. It allows to exponentially amplify a known DNA region with defined edges

(amplicon) in such a way that can be considered a “purification” from the remainder DNA. Moreover, new sequences can be added at the ends of the amplified sequence, enabling cloning into a vector and allowing for site-directed mutagenesis.

2.3.1. Overview of PCR technique—PCR technique was first described in 1986, by Kary Mullis (31). Basically, the reaction consists in repetitions of three defined steps, driven by temperature (Figure 4). In the first step, both strands of DNA are separated by heat-denaturation. In the second step, the reaction medium is cooled to a temperature in which two chemically synthesized oligonucleotides anneal specifically to the flanks of the amplicon. In the third step, the reaction is set to a temperature in which purified DNA polymerase will synthesize complementary strands of the oligonucleotide-flanked region. DNA polymerases can only polymerize DNA in 5' to 3' direction by adding nucleotides to a free 3'-hydroxyl group. For this reason, the oligonucleotides used in this technique are called “primers” (they “prime” polymerase reaction). At the second cycle of amplification (Figure 5), two DNA molecules that comprise exactly the target region to be amplified are generated. As the reaction proceeds, these molecules are amplified geometrically in spite of other-length molecules that are arithmetically amplified (32).

The power of this technique is extraordinary. Prior to its availability, few molecules could be amplified with high specificity to micrograms of DNA. Since its inception multiple applications have been developed, which include DNA cloning and mutagenesis, DNA sequencing, DNA quantification and genotyping, just to name a few.

2.3.2. Factors affecting PCR specificity and efficiency and basic reaction

2.3.2.1. DNA polymerases.: In the first description of the technique, the Klenow fragment of DNA polymerase I from *E. coli* was used. It was denatured in each denaturing step and fresh enzyme had to be added at every cycle. The use of thermophilic DNA polymerases (33) allowed for the automatization of the whole process. The first thermophilic DNA polymerase used in PCR was obtained from *Thermus aquaticus* (Taq DNA polymerase), an aerobic gram-negative bacteria isolated in Yellowstone National Park by Thomas Brock (34). Actually, several companies commercialize thermostable DNA polymerases from *Thermus aquaticus* and other organisms, such as *Pyrococcus furiosus*. Modern DNA polymerases for DNA cloning generally share two common features: (i) they have an improved proofreading activity to minimize misincorporation of nucleotides and (ii) are reversibly inactivated by antibodies, which are inactivated in the first denaturation step (hot-start protocol). This strategy is used to prevent polymerase activity under temperatures lower than melting temperature (t_m , the temperature at which primers anneal to the flanks of the amplicon), that would give unspecific products. Two examples of commercial DNA polymerases suitable for cloning are Fast Start High Fidelity DNA polymerase from Roche and platinum Pfx DNA polymerase from Invitrogen.

In a PCR reaction, there are several factors that affect the specificity and efficiency of thermostable DNA polymerases. Among them, are (i) the polymerase used, (ii) the buffer composition, (iii) the template's source, (iv) the amount of nucleotides and primers, and (v) the thermal cycler program. Accordingly, it is impossible to set conditions that work well for

all PCRs. We recommend choosing the primers following instructions given below and performing a basic PCR reaction. If this reaction does not work (mainly because of a poor efficiency or specificity), it is necessary to test the optimal condition for the reaction.

2.3.2.2. Primers: Several companies commercialize chemically synthesized oligonucleotides at a reasonable price. The choice of a good pair of primers is essential for PCR. There are several factors to consider. The length of the primers should be between 16 and 30 nucleotides and the G/C content should be around 50%. Both primers should have similar T_m s and it is preferable to choose a longer primer than a low T_m primer. T_m depends on length and composition; there are several rules to calculate it. One of them, called “the Wallace rule” (35, 36) which is as follows:

$$T_m(^{\circ}\text{C}) = 2(A+T) + 4(C+G),$$

where (A+T) is the sum of the A and T residues in the oligonucleotide and (C+G) is the sum of C and G residues.

The sequence of the primer should avoid, as far as possible, mono and dinucleotide repetitions, as well as polypurine and polypyrimidine tracts. The 3' last base of the primer must preferably be a G or a C. There should not be secondary structure in the primers, and they should not anneal to each other, especially if this annealing leaves a 3' end that can be a substrate for DNA polymerase. These factors are easily checked with the help of different programs. Among others, Oligo Explorer developed by Teemu Kuulasmaa (<http://www.genelink.com/tools/gl-oe.asp>) is a very simple and useful program freely available online.

Every molecule polymerized in a PCR is flanked by chemically synthesized primers of our choice. This feature allows for insertion of DNA sequences in the primer that will be present in the amplified DNA. One powerful application is the insertion of RE sites in the 5' end of the primers, making much easier the cloning procedure. The addition of non complementary sequences at the 5' end does not significantly affect the T_m of the primer.

A typical PCR reaction contains equimolar amounts of each primer (around 0.2–0.4 μM). Anyway, the concentration of each primer can be increased or decreased (from 0.1 μM to 0.5 μM) to optimize the PCR reaction.

2.3.2.3. Deoxynucleoside triphosphates (dNTPs): In reactions containing MgCl_2 (generally 1.5 mM), a standard PCR mix has equimolar amounts of every dNTP, at concentrations around 200–250 μM . The concentrations of dNTPs and DNA molecules, as well as template and primers, must be related to MgCl_2 concentrations, taking into account the direct chemical interaction between phosphate groups of dNTPs or DNA with divalent cations, such as Mg^{2+} .

2.3.2.4. Divalent cations: Thermostable DNA polymerases need free divalent cations to work. The most widely used cation for PCR is Mg^{2+} (its most common source is MgCl_2). As previously mentioned, the availability of free cations in a PCR reaction depends on the

length and amount of primers, template and nucleotides, because their phosphate groups sequester free cations from the medium. This fact makes it impossible to establish a cation concentration adequate for all PCRs. Commercial PCR kits usually contain two different 10X buffers: (i) one of them contains $MgCl_2$ at concentrations of 15–18 mM (final concentration 1.5–1.8 mM), which are suitable for most PCRs; and (ii) another one that does not contain divalent cations to allow for experimental determination of its optimal concentration.

2.3.2.5. Buffers: PCR reaction needs a buffer to maintain pH. Typically, 10 mM Tris-Cl (pH between 8.3–8.8, at room temperature) is used. At 72 °C (primer extension temperature), pH falls to 7.2, near the optimal for Taq DNA polymerase. PCR buffer also contains 50 mM KCl. The salt concentration affects template denaturation and annealing of primers because it neutralizes the negative charges on the phosphate backbone of DNA, stabilizing the double stranded DNA structure.

2.3.2.5. Template: PCR technique works with small amounts of DNA molecules from any source (either double or single stranded). Some natural sources contain natural inhibitors of the reaction, such as blood or feces (37), which can be easily eliminated through simple DNA purification procedures. Circular DNA amplifies slightly worse than linear, but generally plasmid DNA is shorter and cleaner, producing less unspecific amplifications than cDNA or gDNA. The ideal amount of template is around 5–250 ng for gDNA or cDNA and 100 pg–10 ng for plasmid DNA. Higher concentrations can increase non-specific amplification.

2.3.2.6. Enhancers: Some commercial kits include additives that can enhance PCR efficiency and/or specificity, like dimethylsulfoxide (DMSO), glycerol, ammonium sulfate, detergents and other compounds. Although they are not usually necessary, we recommend following the manufacturer's instructions.

2.3.2.7. Thermal cycling program: As previously mentioned, PCR consists in repetitions of three steps. In the first step, DNA must be heated to be denatured and allow subsequent annealing of the primers. This step is generally performed at a maximum of 94°C during 30 seconds, because Taq DNA polymerase does not resist more than 30 cycles at higher temperatures. When the template is double-stranded, an initial 5' denaturation step (to completely denature DNA sample) is generally added at the beginning of the program.

In the annealing step, temperature is generally set at a few degrees below the theoretical t_m (we recommend 1–3°C). A temperature too close to the t_m does not allow proper annealing, and a temperature too low decreases specificity of the product. Variations in buffer composition, Mg^{2+} , primer or template concentration will affect specificity, and the optimal t_m must be empirically determined.

The optimal temperature for the majority of thermostable DNA polymerases is between 68–78°C. Primer extension step for a PCR performed with Taq DNA polymerase is typically set at 72°C. At this temperature, Taq DNA polymerase can polymerize near to 2000 nucleotides per minute. As a general rule, 1 minute per kilo base of amplicon is necessary to perform accurate primer extension. After the last cycle, a long extension step (10 min) is commonly

added to ensure that all the molecules have been properly polymerized. However, this step is not clearly necessary.

2.3.2.8. PCR optimization: To amplify a DNA fragment, we recommend accurately choosing the primers and performing a basic PCR, following the recommendations given in this section (or in the manufacturer instructions). If the result is not optimal (poor amplification, presence of unspecific or smeared bands), we recommend testing 3–4 different Mg^{2+} concentrations (from 1 to 5 mM) with 3–4 different tms (from t_m to $t_m - 4^{\circ}C$), resulting in 9–16 conditions. This grid generally gives optimal t_m and Mg^{2+} concentration. If none of the different combinations gives a suitable product, it is recommended to set the best couple of conditions and then test different concentrations of each primer. Optimal conditions for the majority of PCRs are established with this method. If it is not your experience, recheck your primers.

2.3.2.9. Basic PCR: Table 1 depicts a summary of the recommended conditions to perform PCR reaction. If the result is not satisfactory, change the used conditions according to recommendations in the previous section.

2.3.3. Site-directed mutagenesis by PCR.—As mentioned above, the artificial sequences introduced in the 5' end of a primer will be present in the amplified molecule. This fact allowed the development of techniques to introduce mutations in a defined position of the amplified sequence (site-directed mutations) (38). The development of site-directed mutagenesis had enormous applications in molecular biology. Point mutations can give information about the function of specific residues and domains in a protein. In this way, proteins can be mutated into inactive forms. Especially informative are the mutations with dominant-negative behavior (7, 8). Furthermore, the introduction of RE sites can be used to create chimeric fusions between two proteins.

Figure 6 shows a simple and effective method to perform PCR-based site-directed mutagenesis as described by Higuchi and co-workers (39). Two independent PCR reactions generate two DNA fragments that overlap in the point of the mutation. One reaction (PCR 1 in the picture) amplifies the amplicon from its 5' end to the mutation point, which is introduced in the primer. The other one (PCR 2) amplifies the amplicon from the point of the mutation to its 3' end, also including the mutation in the primer. When the products of the two reactions are mixed and driven to the melting temperature of the overlapping zone, two kinds of hybrid molecules are formed, one of them hybridizes through the 5' ends and consequently is not a substrate for DNA polymerase, but the second type hybridizes through the 3' ends, leaving the free 3'-OH residues that can prime the polymerization of the rest of the amplicon. This strategy generates a template that has incorporated the mutation and can be amplified in another PCR (PCR 3) with the outer primers already used in PCR 1 and 2.

2.3.4. Protocol: Cloning of mouse Myc Associated Factor X gene (MAX) into pCIG overexpression vector—Specific approaches on cloning techniques (amplifying, cutting and pasting DNA, inserting DNA fragments into vectors, transforming bacteria) were detailed in the previous section. In order to summarize them, we present here a protocol to clone MAX gene into pCIG vector. From initial challenges (such as primer selection) to

latter stages (such as selecting positive bacteria colony), the protocol highlights the steps necessary prior to eukaryotic cell (neuronal) transfection, which are discussed in the third section of this chapter.

1. Primer selection:

Murine MAX mRNA sequence (NCBI accession number: NM_008558) was pasted into Oligo Explorer software. A couple of 20 mer primers comprising exactly the coding sequence (CDS, bases 224–706) were tested. This primer approach presented several problems: The forward primer had a G/C content of 40% whereas the reverse primer had a G/C content of 60 %. This difference in nucleotide composition was transduced to a t_m difference higher than 8°C. Furthermore, thermodynamical study of reverse primer self-annealing revealed the formation of a very stable dimer, which was also a substrate for the polymerase reaction. To avoid this problem, different couples of primers were tested moving the annealing position of both primers few bases outside the amplicon. It is noteworthy that it is not recommended to clone significant parts of untranslated regions (3' or 5' UTRs) of a gene for an overexpression experiment, because they could contain regulatory regions that could affect expression levels and give unexpected results. After few tests, a satisfactory primer couple was found: (i) The forward primer was shifted four bases upstream, giving better features (more G/C content, absence of secondary structures, and a G in its 3' end) and (ii) the reverse primer was shortened to 18 bp, allowing a new t_m of 58°C, avoiding the dangerous self-hybridizing structure observed in its 20 bp form. In order to investigate the potential occurrence of primer dimers (two primers anneal one to the other instead of to the target DNA sequence to be amplified), dimerization calculations were performed. The primer couple did not provide important stable structure, suggesting no possibility of primer dimer formation.

Informatics restriction analysis of the amplicon was confronted with available restriction sites in the MCS of pCIG. XhoI and EcoRI are present in the MCS of pCIG and can be used in the same restriction reaction, because they share common buffer requirements. For this reason, these restriction sites were introduced in the 5' end of the primers previously selected. Commonly, some nucleotides are added at the end of a primer when a RE site is introduced in it, because RE work better if they do not cut in the last bases of the chain. Finally, the chosen primers were:

- Fw MAX XhoI:

GAA CTCGAG GGAAATGAGCGATAACGATG T_m : 58°C XhoI

- Rv MAX EcoRI:

ACC GAATTC TTAGCTGGCCTCCATCCG T_m : 58°C EcoRI

2. A basic PCR was performed using 100 ng of cerebellar granule neuron cDNA. Basic receipt and program given in section 2.3.2.9 were followed. Melting temperature was set at 56°C and primer extension time at 30" (amplicon size:

482 bp). Running of the product in a 1.5% Agarose gel revealed the presence of a moderate-intensity band in the correct weight, with no unspecific extra bands.

3. Band was excised from the gel and DNA was purified in a final volume of 40 μ L with Qiagen's QiaQuick Gel Extraction Kit following the manufacturer's instructions.
4. 15 μ L of the purified band and 1 μ g of pCIG were double-restricted with EcoRI and XhoI with Fermenta's Tango 2x buffer (66 mM Tris-Acetate (pH 7.9 at 37°C), 20 mM Mg-acetate, 132 mM K-acetate, 0.2 mM BSA) and following instructions given in section 2.2.1.1.
5. Bands were excised from the gel and purified again with QiaQuick Gel extraction kit. Again, elution was performed in a 40 μ L volume.
6. 5 μ L of the eluted bands were run on a 1% Agarose gel to quantify the relative quantity of each DNA (since purification procedure gives variable yield, quantification must be done). MAX band was one third intense than pCIG one. As pCIG is 12.86 times larger than MAX, this means MAX is four times more concentrated (in molar terms) than pCIG ($12.86 / 3 = 4.29$).
7. Equal volumes of insert and vector represent a 4.29:1 molar ratio (see section 2.2.2.1) then, ligation reaction was performed as follows: 2 mL of T4 DNA ligase buffer (final concentration: 40 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP), 2 mL of purified restricted pCIG, 2 mL of purified restricted MAX, 0.5 μ L T4-DNA ligase and 13.5 μ L of water were incubated at 17°C overnight. Ligation was repeated adding 2 μ L of water instead of MAX protein (negative control).
8. Ligation reactions were transformed following instructions given in section 2.2.3.1.2 and plates were incubated overnight at 37°C. Around 200 colonies grew in the insert-ligated plate, whereas around 20 colonies grew in negative control plate.
9. To assess insert ligation, 8 colonies were picked. The plasmid DNA was isolated as described in section 2.1.4 and SmaI restriction was performed as described in section 2.2.1.1 (Buffer Tango 1x, 30 °C/1h). The presence of a 300 bp band confirmed insert ligation in all colonies (there is a SmaI site at 300 bp of MAX coding sequence end and at the pCIG's MCS).
10. DNA of a positive colony was large-scale produced and purified (for DNA maxipreparation, see Recommended Reading) and the presence of intact MAX CDS was confirmed by sequencing.

3. Introducing cloned genes into cultured neuronal cells

There are numerous strategies to deliver nucleic acids into cultured cells. They include the transfection with Ca²⁺-phosphate/DNA co-precipitates, lipid-based transfection protocols (lipofection), biolistics, microinjection, electroporation, as well as virus-based infection methods (40, 41). The choice of a specific transfection/infection method is determined by

the experimental goal. With particular emphasis to the transfection of primary cultured neurons, which are post-mitotic cells, most methods remain relatively unsatisfactory with regard to either cytotoxicity or transfection/infection efficiency (42).

This section of the chapter describes the elementary basics and concepts involved in the strategies for delivering nucleic acids into cultured neuronal cells (primary and cell lines), as well as their advantages and drawbacks. A particular emphasis is directed to lipid-based (lipofection) and electroporation-based transfection protocols. For a more rigorous discussion of the topics mentioned here, see the Recommended Reading at the end of the chapter.

3.1. Transfection methods

The methods to deliver nucleic acids into cultured cells fall into three classes: transfection by chemical methods, transfection by physical methods and virus-mediated infection.

3.1.1. Chemical Reagents—DEAE-dextran represents one of the first chemical reagents used to introduce nucleic acids into cultured mammalian cells (43, 44). It is a cationic polymer that associates with the negative charge of nucleic acids. The excess of positive charges allows the complex to come into closer association with the negatively charged cell membrane; its uptake is apparently by endocytosis. This method is useful for delivery of nucleic acids into cells for transient expression (see item 3.2). However, this system is not normally useful for stable transfection studies, where integration of the transferred DNA into the chromosome is required (45). Other synthetic cationic polymers have been used for the transfer of DNA into cells, including polybrene (46), polyethyleneimine (47) and dendrimers (48, 49).

Calcium phosphate co-precipitation represent another interesting technique for the delivery nucleic acids into cultured cells; it became exceedingly popular in the early 1970s (50). The protocol involves mixing DNA with calcium chloride, adding it to a buffered saline/phosphate solution and allowing the mixture to incubate at room temperature. This procedure generates a precipitate that is dispersed onto the cultured cells. The precipitate is taken up by the cells through endocytosis or phagocytosis. The method is commonly used for both transient and stable transfections of a variety of cell types. Complete protocols based on the use of either cationic polymer or calcium phosphate co-precipitation for the delivery of nucleic acids into cultured cells are presented in the book by Sambrook and Russell (Recommended Reading).

Synthetic liposomes are also used to deliver DNA into cells (51). Particularly, the development of synthetic cationic lipids by Felgner and colleagues (52) was a significant progress in liposomal vehicles. The cationic head group of the lipid associates with the negative charges of phosphate groups on the nucleic acids. The methods based on this approach have several advantages such as relatively high efficiency of gene transfer and capability to transfect certain cell types that are resistant to calcium phosphate or DEAE-dextran, such as neuronal cells. Cells transfected by lipofection can be used for transient expression studies and long-term experiments that need the integration of the DNA into the chromosome.

A lipid positively charged at physiological pH is the most frequent synthetic lipid component of liposomes developed for gene delivery (Figure 7). Generally, the cationic lipid is mixed with a neutral lipid such as L-dioleoyl phosphatidylethanolamine (DOPE; Figure 7). The cationic part of the lipid associates with the negative charges of nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex (53). For cultured cells, the positive charge of the liposome/nucleic acid complex allows closer association of the complex with the negatively charged cell membrane, resulting in higher transfection efficiencies. The entrance of the liposome complex into the cell may occur via endocytosis or fusion with the plasma membrane by the lipid moieties of the liposome (54). After cellular internalization, the complexes appear in the endosomes and later in the nucleus (55).

There is a great variety of transfection reagents that use cationic lipids for the delivery of nucleic acids to eukaryotic cells. These include the TransFast™, Tfx™ and Transfectam® Reagents (Promega), Lipofectamine™ LTX Reagent (Invitrogen), and the TransFectin Lipid Reagent (Bio-Rad). The best transfection reagent and conditions for a particular cell type must be determined, because the properties of a particular cell type influence the success of any specific transfection method. Detailed methods based on the use of cationic lipids to deliver nucleic acids into cultured cells are presented in the Recommended Reading. A short lipofection-based protocol to transfect cultured neural cells is presented below.

3.1.1.1. Lipofection protocol: Since many variables affect the efficiency of lipofection, it is suggested that the conditions outlined in the following protocol be used as an initial point for systematic optimization of the system. Alternatively, a protocol recommended by a commercial manufacturer of a particular lipofectant could be used to begin the standardization process. The following protocol is based on the methodologies described by Sambrook and Russell (Recommended Reading) and on the method provided by Invitrogen Corporation (56).

Introducing plasmid DNA into PC12 cells by lipofection

1. PC12 cells are maintained in neurobasal media supplemented with 1% L-Glutamine, 10% FBS (fetal bovine serum) and 5% HS (horse serum), and antibiotics [mixture of penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml)] - in a 5% CO₂ environment.
 - The cells should be around 40–75% confluent at the time of lipofection. If the cells are grown for fewer than 12 hours before transfection, they will not be well anchored to the substratum and are likely to detach during exposure to the lipid.
2. For each transfection sample in 96-well format, dilute 100 ng of plasmid DNA into serum-free medium. Add 0.1 µl of PLUS™ Reagent (Invitrogen) and incubate for 5 min.
3. Lipofectamine™ LTX Reagent (Invitrogen) is then added (transfection should be optimized by titrating the amount of the lipofection reagent) and gently mixed by

pipetting up and down, and the lipid/DNA complex is allowed to form by incubating at room temperature for 30 minutes.

4. Twenty μ l of the DNA-lipid complex is added to each well of the 96-well plates containing cells, which should be in a serum-free medium.
 - It is very important to rinse the cells free of serum before the addition of the lipid-DNA complex. In some cases, serum is a very effective inhibitor of transfection process (57).
5. Incubate the cells for 1–24 hours at 37°C in a 5% CO₂ environment.
6. After the cells have been exposed to the DNA for the appropriate time, wash them three times with serum-free medium. Feed the cells with complete medium and return them to the incubator.

As already mentioned, several variables affect the efficiency of lipofection. Thus, the presented protocol should be used as an initial point for systematic optimization. It is recommended to take into consideration:

- a. The density of the culture: Cell monolayers should be in mid-log phase and should be between 40% and 75% confluent;
- b. Amount of DNA added per dish: Depending on the concentration of the sequences of interest, as little as 50 ng and as much as 40 μ g of DNA might be required to obtain maximum signal for a reporter gene;
- c. Time of exposure of cells to the cationic lipid-DNA complex: Varies from 0.1 to 24 hours;
- d. Purity of the DNA preparation: Whenever possible, the DNA should be dissolved in water rather than buffers containing EDTA. Plasmid preparations used for lipofection should be free of bacterial lipopolysaccharides.

All of these variables must be optimized in order to establish optimal transfection frequencies for a target cell line. Although the current lipofection methods/reagents allow for good transfection efficiencies in neural cell lines, the transfection efficiencies obtained with primary neuronal cells are still low.

3.1.2. Viral Methods—Adenoviral vectors are also useful for gene transfer due to a number of characteristics: (i) they infect a wide range of human cells and can achieve high levels of gene transfer compared to other vectors; (ii) they accommodate relatively large segments of DNA (up to 7.5kb) and transduce these transgenes in nonproliferating cells, such as neurons; and (iii) adenoviral vectors are simple to manipulate using recombinant DNA techniques (58). Other vectors of interest (adeno-associated virus, herpes simplex virus, retroviruses) can infect quiescent cells, and can integrate into the host cell genome to allow stable, long-term expression. Of particular importance, recombinant lentiviral vectors have been described as powerful tools for gene transfer in the central nervous system, showing great potential as a gene therapy strategy for neurological disorders (59).

3.1.3. Physical Methods—Physical methods for delivering nucleic acids were developed in the early 1980s. Direct microinjection into cultured cells is an effective although laborious technique (60). Since the apparatus is costly and the technique extremely laborious, it is not an appropriate method for studies that require a great number of transfected cells.

Another physical method is biolistic particle delivery: particle bombardment. This method is based on high velocity delivery of nucleic acids on microprojectiles to recipient cells by membrane penetration (61). This method has been successfully employed to deliver nucleic acid to cultured cells as well as to cells *in vivo* (62–64).

Electroporation was initially reported for gene transfection into mouse cells (65). This technique is normally used for cell types that are difficult to transfect by other methods. The method is based on perturbing the cell membrane by an electrical pulse, which forms transient pores that allow the entrance of nucleic acids into the cell (66). The technique needs optimization for duration and strength of the pulse for each type of cell used. In addition, electroporation generally requires more cells than chemical methods because of considerable cell death; it is necessary to balance transfection efficiency against cell viability.

To ascertain whether electroporation is a useful method of transfection for a particular cell type/line, it is important to use a range of field strengths and pulse lengths and thus establish conditions that generate the maximal number of transfectants. Several companies that sell electroporation devices offer lists of optimized conditions. However, because of variation in properties between different cultivars of the same cell type/line, it is important for the investigators to confirm that the conditions described in the literature are optimal for cells grown in their laboratory.

Since transfection and cell death are dependent on the field strength (67), it is recommendable to expose aliquots of cells to electric fields of increasing strength with time constants between 20 and 200 milliseconds. For each field strength, measure (i) the number of cells that express the transfected reporter gene and (ii) the proportion of cells that survive exposure to the electric field. Plating efficiency is a more accurate measure of cell survival than staining with vital dyes since, after electroporation, cells can remain permeable to vital dyes such as trypan blue for 1–2 hours. There are other variables that affect the efficiency of electroporation.

- a. The temperature: Electroporation is usually carried out on cells that have been prechilled to 0°C. These cells are maintained at 0°C after electroporation (to maintain the pores in an open position) and are diluted into warm medium for plating (68).
- b. The concentration and the conformation of the DNA: Linear DNA is preferred for stable transformation and circular DNA is preferred for transient transfection (69, 70).

- c. The state of the cells: Best transfections are achieved with cell cultures in the mid-log phase of growth. This represents a limitation for primary cultured neurons.

3.1.3.1. Electroporation protocol: A transfection protocol by electroporation (microporation) in mouse primary cerebellar granule cells follows next. Microporation is an electroporation technology in which a pipette tip is used to provide an electroporation space. Transfections were carried out using a Microporator Pipet-type Electroporation System device (Digital Bio Technology) and the optimal transfection efficiency was achieved by using a single pulse of 1700 V during 20 milliseconds.

1. Primary cerebellar granule cells are obtained from mice according to previously described methods (71);
2. After counting, cells are resuspended in buffer R medium (Digital Bio Technology) at a density of 3×10^7 cells/mL and placed on ice;
3. The plasmid DNA (pCIG-GFP) is added at a concentration of 50 µg/mL (5 µg/ 3×10^6 cells);
4. After mixing, the cell suspension remains on ice and an aliquot of 100 µL (3×10^6 cells) is taken for electroporation, using the pipette device;
5. The microporator pipette is inserted into pipette station and the electroporation is performed using a single pulse of 1,700 V for 20 milliseconds;
 - For optimization, it is important to use a range of field strengths and pulse lengths and thereby establish conditions that generate the maximum number of transfectants for your conditions.
6. After microporation with optimized electric parameter, the cells are immediately transferred into pre-warmed complete medium - Dulbecco's Modified Eagle Medium (DMEM) (25 mM KCl, 31 mM glucose and 0.2 mM glutamine) supplemented with *p*-aminobenzoate, insulin, penicillin and 10% fetal calf serum;
7. The cell suspension (3.2×10^6 cells/mL) is seeded in multiwell plates precoated with poly-D-lysine and incubated at 37°C in a 5% CO₂ environment.
 - A mixture of 5 µM 5-fluoro-2'-deoxyuridine and 20 µM uridine should be added at 24 h after seeding to prevent glial proliferation.
8. Green fluorescent protein (in this case, encoded by the pCIG-GFP) is used as gene reporter (see Figure 8). Transfection efficiency was around 80% in living cells. Cell viability was around 60%.
 - The number of electroporations depends on the number of required plates/wells. Consider to plate cells at a density of 3.2×10^6 cells/mL of the complete medium;
 - As already mentioned, several variables (temperature, DNA concentration, cell type) affect the efficiency of electroporation. Thus,

the above protocol should be used as an initial point for systematic optimization.

3.2. Stable and transient transfection

Following the review of the most common transfection methods, next, we will discuss two different approaches for the delivery of nucleic acids into cultured neuronal cells: transient transfection and stable transfection. In transient transfection, recombinant DNA is introduced in a recipient cell in order to obtain a temporary, but high level expression of the target gene. The transfected DNA is not necessarily integrated into the host chromosome. Transient transfection is practical when a large number of samples are to be analyzed within a short period of time. In general, the cells are harvested at few days after transfection and the resulting lysates can be assayed for expression of the target gene. This approach has been used to overexpress, at few days after transfection, genes that encode proteins involved in neurodegenerative (72) and neuroprotective events (73), as well as neurotransmission (74), antioxidant defenses (75), energetic metabolism (76) and several others physiological events related to the neuronal homeostasis.

Permanent or stable transfection is used to obtain cell lines in which the transfected gene is integrated into chromosomal DNA, allowing for the synthesis of moderate quantities of the target protein. In general, up to 90% of a population of cells expresses the transfected gene in a transient manner. At some point within the first hours after transfection, the transfected DNA undergoes nonhomologous intermolecular recombination and ligation events in order to form a large structure that may integrate into the cellular chromosome (77).

Since the uptake, integration, and expression of DNA are infrequent events, *stable transformants* are generally isolated by selection of the cells that have acquired a new phenotype. This is commonly achieved by co-transfection with a second marker gene, which offers a selectable advantage, such as resistance against a certain antibiotic or drug. In the presence of the toxin in the cell culture, only those cells with the marker gene integrated into their genomes will proliferate and the other cells will die.

The most commonly used selection agent in permanent transfection experiments is Geneticin, also known as G418, which is a toxin that can be neutralized by the product of the neomycin resistant gene (aminoglycoside phosphotransferase) (78). In addition to aminoglycoside phosphotransferase, there are several other selection markers, such as hygromycin-B phosphotransferase (resistance to hygromycin-B) (79), and puromycin-*N*-acetyl transferase (resistance to puromycin) (80). Detailed methods for stable transfection are presented in the book by Sambrook and Russell (Recommended Reading).

3.2.1. Stable Transfectant Protocol—Here, we present an example of a stable transfectant protocol using PC12 cells and G418. The protocol is based on the study by Gollapudi and collaborators (81), which delves into the use of stable transfection of PC12 cells with the estrogen receptor (ER α) in order to detect protective effects.

1. PC12 cells are maintained in a “complete medium” - DMEM (Dulbecco’s modified Eagle medium) containing 5% FBS (fetal bovine serum) and 10% HS

(horse serum), 5 mM glutamine, and antibiotics [mixture of penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml)] - in a 5% CO₂ environment.

2. The cells are seeded at a concentration of 1.5×10^6 cells per well into collagen-polylysine-coated 6-well culture dishes. They are allowed to adhere overnight, and the next day washed twice with serum-free DMEM.
3. The introduction of the plasmid DNA is mediated by lipofection. Transfection solution containing lipofection reagent and DNA (vector alone or vector ligated with ER cDNA) is carefully overlaid and incubated with the cells for 5 hr.
 - For details on DNA transfection mediated by lipofection, please see item 3.1.2.
 - The used plasmid DNA (vector pcDNA3) encodes the aminoglycoside phosphotransferase gene, which confers resistance to geneticin (G418).
4. After lipofection, cells are washed three times with serum-free medium. Next, complete medium is added and the cells are incubated for 4 days, with media being replaced every day.
5. To isolate stable transfectants, cells are trypsinized and replated in the selective medium (complete medium containing 500 µg/ml of G418).
6. Change this medium every 2–4 days for 2–3 weeks to remove the debris of death cells and to allow colonies of resistant cells to grow.
7. Individual colonies can be isolated by cloning cylinders, selected and transferred to multiwell plates for further propagation in the presence of selective medium.
 - Individual cells that survive the drug treatment expand into clonal groups that can be individually propagated and characterized.
 - To standardize a protocol, it is necessary to determine the killing concentration (kill curve) of the selective drug prior to transfection (82).

3.3. Analysis of transfection efficiency

As already mentioned, after transfection of cultured neuronal cells with a specific DNA sequence, it is important to determine the percentage of cells that have received and are expressing the foreign DNA. In addition, it is important to quantify the relative or absolute increases in the expression of the protein encoded by the transfected gene in the whole sample.

To quantify the expression of a given protein encoded by the transfected gene, semi-quantitative and quantitative methods can be used. Immunocytochemistry (83), western blot (1), flow cytometric analyses (84) and protein activity (85) can give a general view of the expression of the transfected gene.

In order to determine the percentage of transfected cells that have received and are expressing the foreign DNA sequence, a reporter gene can be used. The reporter gene can be

present on the same vector as the gene of interest or it can be localized on a separate plasmid. As already stated, a convenient reporter for monitoring transfection efficiency is the green fluorescent protein (GFP; see item 2.1.1). The typical image of cultured cells expressing GFP is presented in Figure 8. For quantitative evaluations on the transfection efficiency, counts of the number of nuclei (DAPI) and cells expressing GFP should be conducted, deriving a percentage of the transfection efficiency. Notably, there are specific antibodies for GFP which allow its detection in cells even when low expression rates are achieved.

4. Applications of gene transfection to study neuropathology and neurotoxicity

Advances in molecular cloning techniques have revolutionized over the last 2–3 decades, resulting in an explosion in the number of both *in vitro* and *in vivo* studies on the consequences of overexpression or deletion of specific proteins and their role in neurodegeneration or neurotoxicity (86–90). It is noteworthy that in contrast to the huge number of studies on the role of specific proteins in pathways that lead to cell death or dysfunctioning in chronic neurodegenerative diseases (i.e. Alzheimer and Parkinson's diseases), the number of studies on the modulatory role of specific proteins in neurotoxicity elicited by environmental neurotoxicants is relatively limited (1, 91, 92). The tools/protocols described in the previous sections of this chapter and the illustrative studies cited herein intend to provide basic information on how these techniques can potentially be used to advance the knowledge in the neuroscience field. We suggest that the use of the aforementioned techniques can simplify the task of studying complex signaling pathways upon exposure to different toxicants in the whole cell (neuronal) milieu. With a particular emphasis on the neurotoxicity induced by xenobiotics, genetically transformed neuronal cells can be considered a powerful tool for screening and identifying specific molecular targets and deciphering complex cascades of molecular events involved in either neurotoxic or neuroprotective events (1, 91).

4.1. Neurodegenerative diseases

Several laboratories have taken advantage of genetically modifying different types of organisms, specific mammalian brain areas and/or cultured neuronal cells to clarify the role of putative key proteins as modulatory factors of different neuropathological conditions (73–75, 81, 86–90, 93). Next, we illustrate several examples on how the transfection of cultured neuronal cells can assist in elucidating the role played by specific proteins in neurodegenerative diseases and neurotoxicity induced by environmental neurotoxicants. Because of the large number of studies on these topics and due to space limitations, particular emphasis on Parkinson's disease and methylmercury-induced neurotoxicity is presented.

4.1.1. The case of Parkinson's Disease (PD)—The intracellular deposition of misfolded proteins is believed to participate in age-related neurodegenerative diseases (93–95). For instance, PD is associated with degeneration of dopaminergic neurons located in the *substantia nigra* and with the presence of inclusion bodies containing alpha-synuclein (89,

95). The exact role of alpha-synuclein misfolding in the neurotoxicity of PD is still unknown. However, *in vitro* and *in vivo* transfection models of neuronal cells overexpressing wild type or mutated alpha-synuclein have indicated that its aggregation can modify different intracellular processes and contribute to neuronal death (89, 96). Consistent with these observations, Lee and co-workers (97) have demonstrated that dorsal root ganglia neurons overexpressing alpha-synuclein exhibit microtubule dysfunction, neuritic degeneration, trafficking defects and Golgi fragmentation, which are common features in other human neurodegenerative diseases. Hsu et al. have investigated the effects of alpha-synuclein overexpression in a hypothalamic neuronal cell line and in an analogous fashion observed that its overexpression resulted in formation of alpha-synuclein-immunopositive aggregates and mitochondrial dysfunction associated with increased formation of reactive oxygen species. They have also demonstrated that pretreatment with vitamin E ameliorated the alpha-synuclein-induced neuronal toxicity (98).

Another protein that is involved in PD etiology is parkin (89, 96) and data obtained with transfected neuronal cells have been fundamental in elucidating the antagonistic effect of wild-type parkin and the stimulatory effect of its mutants to PD development. For instance, Hyun et al. (99) have reported that neuronal toxicity and oxidative stress caused by different factors (serum withdrawal, H₂O₂, MPP(+), or 4-hydroxy-2-trans-nonanal) were attenuated by wild-type parkin overexpression. In contrast, overexpression of the mutant parkins increased cell death and oxidative stress markers in response to these stressors. Consequently, it was concluded that parkin mutation in *substantia nigra* in PD may increase neuronal vulnerability to a range of toxic insults, which could facilitate disease initiation and progression (99). In line with this *in vitro* study, *in vivo* rodent transgenic models have indicated a neuroprotective role for wild-type parkin, where overexpression of the gene decreased the responsiveness to alpha-synuclein-induced neuropathology (100).

4.2. Mechanisms of neurotoxicity

Neurodegeneration is a complex process and represents a hallmark in a variety of acute and chronic human diseases (94). Although the cascade of events that occur in each disease can be unique, from the point of view of gross behavioral, cellular and molecular outcomes, the pathways that lead to the various diseases can have overlapping molecular mechanisms. For instance, oxidative stress is believed to underlie neuronal dysfunction in many neurodegenerative disorders (100–102), including those caused by environmental toxicants (91, 103). In this regard, a variety of studies have taken advantage of neuronal transfection with potentially toxic or protective key proteins to demonstrate that oxidative stress and apoptosis can be a common modulators of neurodegeneration. For instance, primary cultures of neocortical neurons obtained from transgenic mice overexpressing human Bcl-2 under the control of neuron-specific enolase promoter were more resistant to the pro-oxidant and dopaminergic neurotoxin 6-OHDA than neurons from normal mice (104). These results indicate that the anti-apoptotic Bcl-2 protein can modulate oxidative stress and dopaminergic cell death. In the same vein, overexpression Bcl-2 protects PC12 cells from 3-nitropropionic acid-induced hydrogen peroxide production, mitochondrial DNA damage and cell death (105). However, Bcl-2 protection against apoptotic cell death and increased oxidative stress in dopaminergic PC12 cells was dependent on a definite level of expression. In fact,

induction of high-level expression of Bcl-2 in PC12 cells resulted in generation of oxidative stress and cessation of growth by cell cycle arrest, suggesting that Bcl-2 expression is beneficial only in a limited range and that its high-level expression can cause neurotoxicity (106).

The relationship between environmental toxicants and neuropathological conditions represents an important topic that deserves particular consideration when neurotoxicity and neuropathology are analyzed from a mechanistic point of view. In fact, although genetic research has shown that particular gene variations predispose some individuals to neuropathologies, the role of the environment in the etiology of such conditions has become clearer (107). Of particular importance, the exposure of brain to environmental toxicants can trigger the activation of cytotoxic pathways that partially overlaps with those triggered by neurodegenerative diseases (108–110). Environmental toxicants, such as MPPT, 3-nitropropionic acid and toxic metals can cause oxidative stress and mitochondrial dysfunction (103, 108–111). In this regard, methylmercury (MeHg) is an environmental neurotoxicant that seems to cause neurotoxicity via disruption of multiple targets, which will culminate in mitochondrial dysfunction and oxidative stress (103). However, the exact role of specific targets in MeHg neurotoxicity is still not fully defined. We have observed that MeHg-induced neuronal cell death in cultured mouse cerebellar granule cells was preceded by a marked inhibition of the antioxidant enzyme glutathione peroxidase 1 (GPx1) (1) and that the overexpression of GPx-1 prevented MeHg-induced neuronal death. These results indicate that GPx-1 is an important molecular target involved in MeHg-induced neurotoxicity and reinforce the view that peroxides and oxidative stress are fundamental factors in MeHg toxicity. In accordance with this, Nrf2 overexpression attenuated MeHg-induced cytotoxicity in SH-SY5Y cells (112), which is in line with data showing a protective role for Nrf2 activation in astrocytes exposed to MeHg (113). Collectively, these data can help to explain the earlier demonstration that overexpression of metallothionein in cultured astrocytes affords protection against the cytotoxic effects of MeHg (114), particularly, in view of the demonstration that metallothionein protected SH-SY5Y cells from the neurotoxic effects caused by 6-OHDA via activation of the Nrf-2 signaling pathway (115).

The illustrative and simplified examples presented above support an important role for oxidative stress and apoptosis in cell death caused by different neurotoxic agents and also an important role for antioxidant or antiapoptotic proteins as antagonists of neuropathology in different *in vitro* models. The development of transgenic cells that overexpress key proteins, such as GPx1, parkin and alpha-synuclein, among others, is a valuable tool for determining the molecular mechanisms involved in different neuropathological conditions. Furthermore, transgenic cells could be used to help neurotoxicologists in the difficult task of screening environmental toxicants with potential hazard for predisposition for neurodegenerative diseases in humans (116).

Acknowledgments

The authors would like to thank to the agencies that support their research activities (in alphabetical order): Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq and INCT for Excitotoxicity and Neuroprotection-MCT/CNPq, Brazil (Marcelo Farina and João B. T. Rocha); Fundação de Amparo à Pesquisa do Estado de Santa Catarina, Brazil (Marcelo Farina); NIH ES10563 and ES 07331 (Michael Aschner); Spanish

Ministry of Education Grant BFU2008-02424/BFI (Sebastián Pons). Jordi Berenguer is a FPI fellow in Sebastián Pons laboratory (fellowship also supported by the Spanish Ministry of Education Grant BFU2008-02424/BFI).

References:

1. Farina M, Campos F, Vendrell I, Berenguer J, Barzi M, Pons S, and Suñol C (2009) Probuco increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells. *Toxicol Sci* 112, 416–26. [PubMed: 19770487]
2. Anderson S, Davis DL, Dahlbäck H, Jörnvall H, and Russell DW (1989) Cloning, Structure and Expression of the Mitochondrial Cytochrome p-450 Sterol 26-Hydrolase, a Bile Acid Biosynthetic Enzyme. *J Biol Chem* 264, 8222–29. [PubMed: 2722778]
3. Boshart M, Weber F, Jahn G, Dorsch-Häsler K, Fleckenstein B, and Schafner W (1985). A Very strong enhancer is located upstream of an immediate early gene of human Cytomegalovirus. *Cell* 41, 521–30. [PubMed: 2985280]
4. Nelson JA, Reynolds-Kohler C, and Smith BA (1987). Negative and positive regulation by a short segment in the 5' flanking region of the human cytomegalovirus major immediate-early gene. *Molec Cell Biol* 7, 4125–29. [PubMed: 2828927]
5. Cereghini S, Herbolme P, Jouanneau J, Saragosti S, Katinka M, Bourachot B, de Crombrughe B, and Yaniv M (1983) Structure and function of the promoter-enhancer region of Polyoma and SV40. *Cold Spring Harb Symp Quant Biol* 47, 935–44. [PubMed: 6305593]
6. Cheng L, Ziegelhoffer PR, and Yang NS (1993) In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by particle bombardment. *Proc Nat Acad Sci USA* 90, 4455–59. [PubMed: 8506285]
7. Herskowitz I (1987) Functional inactivation of genes by dominant negative mutations. *Nature* 329, 219–22. [PubMed: 2442619]
8. Sheppard D (1994) Dominant negative mutants: tools for the study of protein function in vitro and in vivo. *Am J Respir Cell Mol Biol* 11, 1–6. [PubMed: 8018332]
9. Wood KV, de Wet JR, Dewji N, and DeLuca M (1984) Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns. *Biochem Biophys Res Commun* 124, 592–6. [PubMed: 6208909]
10. de Wet JR, Wood KV, Helinski DR, and DeLuca M (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. *Proc Natl Acad Sci USA* 82, 7870–3. [PubMed: 3906652]
11. Vieites JM, Navarro-García F, Pérez-Díaz R, Pla J, and Nombela C (1994) Expression and in vivo determination of firefly luciferase as gene reporter in *Saccharomyces cerevisiae*. *Yeast* 10, 1321–27. [PubMed: 7900421]
12. Gailey PC, Miller EJ, and Griffin GD (1997) Low-cost system for real-time monitoring of luciferase gene expression. *BioTechniques* 22, 528–34. [PubMed: 9067033]
13. Cohen SN, Chang AC, Boyer HW, and Helling RB (1973) Construction of biologically functional bacterial plasmids in vitro. *Proc Nat Acad Sci USA* 70, 3240–4. [PubMed: 4594039]
14. Dussoix D, and Arber W (1962) Host specificity of DNA produced by *Escherichia coli*. I. Host controlled modification of bacteriophage lambda. *J Mol Biol* 5, 18–36. [PubMed: 13862047]
15. Dussoix D, and Arber W (1962) Host specificity of DNA produced by *Escherichia coli*. II. Control over acceptance of DNA from infecting phage lambda. *J Mol Biol* 5, 37–49. [PubMed: 13888713]
16. Boyer H (1964) Genetic control of restriction and modification in *Escherichia coli*. *J Bacteriol* 88, 1652–60. [PubMed: 14240953]
17. Meselson M, and Yuan R (1968) DNA restriction enzyme from *E. coli*. *Nature* 217, 1110–14. [PubMed: 4868368]
18. Arber W, and Linn S (1969) DNA Modification and Restriction. *Ann. Rev Biochem* 38, 467–500. [PubMed: 4897066]
19. Smith HO, and Wilcox KW (1970) A restriction enzyme from *Haemophilus influenzae*. I. Purification and general properties. *J Mol Biol* 51, 379–91. [PubMed: 5312500]
20. Kelly TJ, and Smith HO (1970) A restriction enzyme from *Haemophilus influenzae*. II. Base sequence of the recognition site. *J Mol Biol* 51, 393–409. [PubMed: 5312501]

21. Hattman S, Brooks JE, and Masurekar M (1978) Sequence specificity of the P1 modification methylase (M.Eco P1) and the DNA methylase (M.Eco dam) controlled by the Escherichia coli dam gene. J Mol Biol 126, 367–80. [PubMed: 370402]
22. Wilson GG (1991) Organization of restriction-modification systems. Nucleic Acids Res 19, 2539–66. [PubMed: 2041731]
23. Tomkinson AE, Vijayakumar S, Pascal JM, and Ellenberger T (2006) DNA ligases: structure, reaction mechanism, and function. Chem Rev 106, 687–99. [PubMed: 16464020]
24. Olivera BM, Hall ZW, and Lehman IR (1968) Enzymatic joining of polynucleotides. V. A DNA adenylate intermediate in the polynucleotide joining reaction. Proc Nat Acad Sci USA 61, 237–44 [PubMed: 4301588]
25. Gumport RI, and Lehman IR (1971) Structure of the DNA ligase-adenylate intermediate: lysine (epsilon-amino)-linked adenosine monophosphoramidate. Proc Nat Acad Sci USA 68, 2559–63. [PubMed: 4944632]
26. Griffith F (1928) The significance of pneumococcal types. J Hyg 27, 113–159 [PubMed: 20474956]
27. Mandel M, and Higa A (1970) Calcium-dependent bacteriophage DNA infection. J Mol Biol 53, 159–62 [PubMed: 4922220]
28. Cohen SN, Chang ACY, and Hsu L (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-Factor DNA. Proc Nat Acad Sci USA 69, 2110–14 [PubMed: 4559594]
29. Klebe RJ, Harriss JV, Sharp ZD, and Douglas MG (1983) A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene 25, 333–41. [PubMed: 6363214]
30. Chung CT, and Miller RH (1988) A rapid and convenient method for the preparation and storage of competent bacterial cells. DNA 7, 609–15 [PubMed: 3229285]
31. Mullis KB, Faloona F, Scharf S, Saiki R, Horn G, and Erlich H (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol 51, 263–73. [PubMed: 3472723]
32. Mullis KB, and Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155, 335–50 [PubMed: 3431465]
33. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, and Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487–91
34. Brock TD, and Freeze H (1969) *Thermus aquaticus* gen. n. and sp. n., a nonsporulating extreme thermophile. J Bacteriol 98, 289–97. [PubMed: 5781580]
35. Suggs SV, Wallace RB, Hirose T, Kawashima EH, and Itakura K (1981) Use of synthetic oligonucleotides as hybridization probes: isolation of cloned cDNA sequences for human beta 2-microglobulin. Proc Natl Acad Sci USA 78, 6613–7 [PubMed: 6171820]
36. Thein SL, and Wallace RB (1986) The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. Chapter in Human genetic diseases: a practical approach, ed. Davies KE, IRL Press, Oxford, UK, 33–50.
37. Bickley J, and Hopkins D (1999) Inhibitors and enhancers of PCR. Chapter in Analytical Molecular Biology: Quality and Validation, eds Saunders GC, and Parkes HC, Royal Society of Chemistry, London, 81–102.
38. Kunkel TA, Roberts JD, and Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol 154, 367–82. [PubMed: 3323813]
39. Higuchi R, Krummel B, and Saiki RK (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 16, 7351–67 [PubMed: 3045756]
40. Washbourne P, and McAllister AK (2002) Techniques for gene transfer into neurons. Curr Opin Neurobiol 12, 566–73. [PubMed: 12367637]
41. Dahm R, Zeitelhofer M, Gotze B, Kiebler MA, and Macchi P (2008) Visualizing mRNA localization and local protein translation in neurons. Methods Cell Biol 85, 293–327. [PubMed: 18155468]

42. Goetze B, Grunewald B, Baldassa S, and Kiebler M (2004) Chemically controlled formation of a DNA/calcium phosphate coprecipitate: application for transfection of mature hippocampal neurons. *J Neurobiol* 60, 517–25. [PubMed: 15307155]
43. Pagano JS, and Vaheiri A (1965) Enhancement of infectivity of poliovirus RNA with diethylaminoethyl-dextran (DEAE-D). *Arch Gesamte Virusforsch* 17, 456–64. [PubMed: 4286824]
44. McCutchan JH, and Pagano JS (1968) Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J Natl Cancer Inst* 41, 351–7. [PubMed: 4299537]
45. Gluzman Y (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* 23, 175–82. [PubMed: 6260373]
46. Kawai S, and Nishizawa M (1984) New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol Cell Biol* 4, 1172–4. [PubMed: 6330534]
47. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, and Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* 92, 7297–301. [PubMed: 7638184]
48. Haensler J, and Szoka FC Jr. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug Chem* 4, 372–9. [PubMed: 8274523]
49. Kukowska-Latallo JF, Bielinska AU, Johnson J, Spindler R, Tomalia DA, and Baker JR Jr. (1996) Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc Natl Acad Sci U S A* 93, 4897–902. [PubMed: 8643500]
50. Graham FL, and van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456–67. [PubMed: 4705382]
51. Fraley R, and Papahadjopoulos D (1982) Liposomes: the development of a new carrier system for introducing nucleic acid into plant and animal cells. *Curr Top Microbiol Immunol* 96, 171–91. [PubMed: 6173163]
52. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, and Danielsen M (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* 84, 7413–7. [PubMed: 2823261]
53. Labat-Moleur F, Steffan AM, Brisson C, Perron H, Feugeas O, Furstemberger P, Oberling F, Brambilla E, and Behr JP (1996) An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther* 3, 1010–7. [PubMed: 9044741]
54. Gao X, and Huang L (1995) Cationic liposome-mediated gene transfer. *Gene Ther* 2, 710–22. [PubMed: 8750010]
55. Farhood H, Bottega R, Epand RM, and Huang L (1992) Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity. *Biochim Biophys Acta* 1111, 239–46. [PubMed: 1420259]
56. Invitrogen Corporation. BioTechniques Protocol Guide (2009) Transfection of PC12 Cells With Lipofectamine™ LTX Does Not Affect Their Ability to Differentiate Upon NGF Stimulation, page 33, doi: 10.2144/000113028.
57. Felgner PL, Holm M, and Chan H (1989) Cationic liposome mediated transfection. *Proc West Pharmacol Soc* 32, 115–21. [PubMed: 2780576]
58. Vorburger SA, and Hunt KK (2002) Adenoviral gene therapy. *Oncologist* 7, 46–59.
59. Lundberg C, Björklund T, Carlsson T, Jakobsson J, Hantraye P, Déglon N, Kirik D (2008) Applications of lentiviral vectors for biology and gene therapy of neurological disorders. *Curr Gene Ther* 8, 461–73. [PubMed: 19075629]
60. Capecchi MR (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* 22, 479–88. [PubMed: 6256082]
61. Ye GN, Daniell H, and Sanford JC (1990) Optimization of delivery of foreign DNA into higher-plant chloroplasts. *Plant Mol Biol* 15, 809–19. [PubMed: 2103474]
62. Klein TM, Fromm M, Weissinger A, Tomes D, Schaaf S, Sletten M, and Sanford JC (1988) Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. *Proc Natl Acad Sci U S A* 85, 4305–4309. [PubMed: 16593942]

63. Burkholder JK, Decker J, and Yang NS (1993) Rapid transgene expression in lymphocyte and macrophage primary cultures after particle bombardment-mediated gene transfer. *J Immunol Methods* 165, 149–56. [PubMed: 8228267]
64. Ogura R, Matsuo N, Wako N, Tanaka T, Ono S, and Hiratsuka K (2005) Multi-color luciferases as reporters for monitoring transient gene expression in higher plants. *Plant Biotechnology* 22, 151–5.
65. Wong TK, and Neumann E (1982) Electric field mediated gene transfer. *Biochem Biophys Res Commun* 107, 584–7. [PubMed: 7126230]
66. Shigekawa K, and Dower WJ (1988) Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. *Biotechniques* 6, 742–51. [PubMed: 3273636]
67. Chu G, Hayakawa H, and Berg P (1987) Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res* 15, 1311–26. [PubMed: 3029703]
68. Rabussay D, Uher L, Bates G, and Piastuch W (1987) Electroporation of mammalian and plant cells. *Focus (Life Technologies)* 9, 1–3.
69. Neumann E, Schaefer-Ridder M, Wang Y, and Hofschneider PH (1982) Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *Embo J* 1, 841–5. [PubMed: 6329708]
70. Potter H, Weir L, and Leder P (1984) Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc Natl Acad Sci U S A* 81, 7161–5. [PubMed: 6438633]
71. Alvarez-Rodriguez R, Barzi M, Berenguer J, and Pons S (2007) Bone morphogenetic protein 2 opposes Shh-mediated proliferation in cerebellar granule cells through a TIEG-1-based regulation of Nmyc. *J Biol Chem* 282, 37170–80. [PubMed: 17951258]
72. Ting JT, Kelley BG, Lambert TJ, Cook DG, and Sullivan JM (2007) Amyloid precursor protein overexpression depresses excitatory transmission through both presynaptic and postsynaptic mechanisms. *Proc Natl Acad Sci U S A* 104, 353–8. [PubMed: 17185415]
73. Monje ML, Phillips R, and Sapolsky R (2001) Calbindin overexpression buffers hippocampal cultures from the energetic impairments caused by glutamate. *Brain Res* 911, 37–42. [PubMed: 11489442]
74. Rintoul GL, Raymond LA, and Baimbridge KG (2001) Calcium buffering and protection from excitotoxic cell death by exogenous calbindin-D28k in HEK 293 cells. *Cell Calcium* 29, 277–87. [PubMed: 11243935]
75. Haque ME, Asanuma M, Higashi Y, Miyazaki I, Tanaka K, and Ogawa N (2003) Overexpression of Cu-Zn superoxide dismutase protects neuroblastoma cells against dopamine cytotoxicity accompanied by increase in their glutathione level. *Neurosci Res* 47, 31–7. [PubMed: 12941444]
76. Gimenez-Cassina A, Lim F, Cerrato T, Palomo GM, and Diaz-Nido J (2009) Mitochondrial hexokinase II promotes neuronal survival and acts downstream of glycogen synthase kinase-3. *J Biol Chem* 284, 3001–11. [PubMed: 19033437]
77. Perucho M, Hanahan D, and Wigler M (1980) Genetic and physical linkage of exogenous sequences in transformed cells. *Cell* 22, 309–17. [PubMed: 6253083]
78. Maisonneuve C, and Echalié G (1986) Stable transformation of *Drosophila* Kc cells to antibiotic resistance with the bacterial neomycin resistance gene. *FEBS Lett* 197, 45–9. [PubMed: 3005047]
79. Blochlinger K, and Diggelmann H (1984) Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. *Mol Cell Biol* 4, 2929–31. [PubMed: 6098829]
80. Vara JA, Portela A, Ortin J, and Jimenez A (1986) Expression in mammalian cells of a gene from *Streptomyces alboniger* conferring puromycin resistance. *Nucleic Acids Res* 14, 4617–24. [PubMed: 3714487]
81. Gollapudi L, and Oblinger MM (1999) Stable transfection of PC12 cells with estrogen receptor (ER α): protective effects of estrogen on cell survival after serum deprivation. *J Neurosci Res* 56, 99–108. [PubMed: 10213481]
82. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K (1999) *Short Protocols in Molecular Biology*, 4th Edition (Paperback). John Wiley & Sons.

83. Domingues A, Almeida S, da Cruz e Silva EF, Oliveira CR, and Rego AC (2007) Toxicity of beta-amyloid in HEK293 cells expressing NR1/NR2A or NR1/NR2B N-methyl-D-aspartate receptor subunits. *Neurochem Int* 50, 872–80. [PubMed: 17403555]
84. Chong KW, Lee AY, Koay ES, Seet SJ, and Cheung NS (2006) pH dependent high transfection efficiency of mouse neuroblastomas using TransFectin. *J Neurosci Methods* 158, 56–63. [PubMed: 16814394]
85. Huang RQ, Qu YH, Ke WL, Zhu JH, Pei YY, and Jiang C (2007) Efficient gene delivery targeted to the brain using a transferrin-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. *Faseb J* 21, 1117–25. [PubMed: 17218540]
86. Gabizon R and Taraboulos A (1997) Of mice and (mad) cows-transgenic mice help to understand prions. *Trends Genet.* 13, 264–9. [PubMed: 9242048]
87. Kirik D and Björklund A (2003) Modeling CNS neurodegeneration by overexpression of disease-causing proteins using viral vectors. *Trends Neurosci.* 26, 386–92. [PubMed: 12850435]
88. Kobayashi Y and Sobue G (2001) Protective effect of chaperones on polyglutamine diseases. *Brain Res. Bull* 56, 165–8. [PubMed: 11719246]
89. Moore DJ, West AB, Dawson VL and Dawson TM (2005) Molecular pathophysiology of Parkinson's disease. *Annu. Rev. Neurosci* 28, 57–87. [PubMed: 16022590]
90. Rockenstein E, Crews L and Masliah E (2007) Transgenic animal models of neurodegenerative diseases and their application to treatment development. *Adv. Drug. Deliv. Rev* 59, 1093–102. [PubMed: 17869376]
91. Kitazawa M, Anantharam V and Kanthasamy AG (2003) Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase C δ in dopaminergic cells: Relevance to oxidative stress and dopaminergic degeneration. *Neuroscience* 119, 945–64. [PubMed: 12831855]
92. Thiruchelvam M, Prokopenko O, Cory-Slechta DA, Richfield EK, Buckley B and Mirochnitchenko O (2005) Overexpression of superoxide dismutase or glutathione peroxidase protects against the paraquat plus maneb-induced Parkinson disease phenotype. *J. Biol. Chem* 280, 22530–9. [PubMed: 15824117]
93. Zoghbi HY and Botas J (2002) Mouse and fly models of neurodegeneration. *Trends Genet.* 18, 463–71. [PubMed: 12175807]
94. Jellinger KA (2009) Recent advances in our understanding of neurodegeneration. *J. Neural. Transm* 116, 1111–62. [PubMed: 19707851]
95. Lee SJ (2003) alpha-synuclein aggregation: a link between mitochondrial defects and Parkinson's disease? *Antioxid. Redox Signal.* 5, 337–48. [PubMed: 12880487]
96. Di Napoli M (2009) New molecular avenues in Parkinson's disease therapy. *New molecular avenues in Parkinson's disease therapy. Curr. Top. Med. Chem* 9, 913–48. [PubMed: 19754406]
97. Lee HJ, Khoshaghideh F, Lee S and Lee SJ (2006) Impairment of microtubule-dependent trafficking by overexpression of alpha-synuclein. *Eur. J. Neurosci* 24, 3153–62. [PubMed: 17156376]
98. Hsu LJ, Sagara Y, Arroyo A, Rockenstein E, Sisk A, Mallory M, Wong J, Takenouchi T, Hashimoto M and Masliah E (2000) alpha-synuclein promotes mitochondrial deficit and oxidative stress. *Am. J. Pathol* 157, 401–10. [PubMed: 10934145]
99. Hyun DH, Lee M, Halliwell B and Jenner P (2005) Effect of overexpression of wild-type or mutant parkin on the cellular response induced by toxic insults. *J. Neurosci. Res* 82, 232–44. [PubMed: 16130151]
100. Lo Bianco C, Schneider BL, Bauer M, Sajadi A, Brice A, Iwatsubo T and Aebischer P (2004) Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an alpha-synuclein rat model of Parkinson's disease. *Proc. Nat. Acad Sci. USA* 101, 17510–5. [PubMed: 15576511]
101. Reynolds A, Laurie C, Mosley RL and Gendelman HE. (2007) Oxidative stress and the pathogenesis of neurodegenerative disorders. *Int. Rev. Neurobiol* 82, 297–325. [PubMed: 17678968]
102. Moreira PI, Zhu X, Wang X, Lee H. g., Nunomura A, Petersen RB, Perry G, (2010) Mitochondria: A therapeutic target in neurodegeneration *Biochim. Biophys. Acta – Mol. Bais Dis* 1802, 212–20.

103. Aschner M, Syversen T, Souza DO, Rocha JBT and Farina M (2007) Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. *Brazilian J. Med. Biol. Res* 40, 285–91.
104. Offen D, Beart PM, Cheung NS, Pascoe CJ, Hochman A, Gorodin S, Melamed E, Bernard R and Bernard O (1998) Transgenic mice expressing human Bcl-2 in their neurons are resistant to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. *Proc. Nat. Acad. Sci. USA* 95, 6789–94.
105. Mandavilli BS, Boldogh I and Van Houten B (2005) 3-Nitropropionic acid-induced hydrogen peroxide, mitochondrial DNA damage, and cell death are attenuated by Bcl-2 overexpression in PC12 cells. *Mol. Brain Res.* 133, 215–23. [PubMed: 15710238]
106. Seyfried J, Evert BO, Schwarz CS, Schaupp M, Schulz JB, Klockgether T and Wüllner U (2003) Gene dosage-dependent effects of bcl-2 expression on cellular survival and redox status. *Free Radic Biol Med.* 34, 1517–30. [PubMed: 12788472]
107. Schmidt CW (2007) Environmental connections: a deeper look into mental illness. *Environ Health Perspect.* 115, A404, A406–10.
108. Kotake Y and Ohta S (2003) MPP plus analogs acting on mitochondria and inducing neurodegeneration. *Cur. Med. Chem* 10, 2507–2516.
109. do Nascimento JLM, Oliveira KRM, Crespo-Lopez ME, Macchi BM, Maues LAL, Pinheiro MDN, Silveira LCL, Herculano AM (2008) Methylmercury neurotoxicity & antioxidant defenses. *Ind. J. Med. Res* 128, 373–82.
110. Quintanilla RA and Johnson GVW (2009) Role of mitochondrial dysfunction in the pathogenesis of Huntington's disease. *Brain Res. Bull* 80, 242–47. [PubMed: 19622387]
111. Valko M, Rhodes CJ, Moncol J, Izakovic M and Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem. Biol. Int* 160, 1–40.
112. Toyama T, Sumi D, Shinkai Y, Yasutake A, Taguchi K, Tong KI, Yamamoto M and Kumagai Y (2007) Cytoprotective role of Nrf2/Keap1 system in methylmercury toxicity. *Biochem. Biophys. Res. Com* 363, 645–50. [PubMed: 17904103]
113. Wang L, Jiang HY, Yin ZB, Aschner M and Cai JY (2009) Methylmercury Toxicity and Nrf2-dependent Detoxification in Astrocytes. *Toxicol. Sci* 107, 135–43. [PubMed: 18815141]
114. Yao CP, Allen JW, Conklin DR and Aschner M (1999) *Brain Res.* 818, 414–20. [PubMed: 10082827]
115. Hwang YP, Kim HG, Han EH and Jeong HG (2008) Metallothionein-III protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in a PI3K and ERK/Nrf2-dependent manner. *Toxicol Appl Pharmacol* 231, 318–27. [PubMed: 18554677]
116. Forsby A, Bal-Price AK, Coecke S, Fabre N, Gustafsson H, Honegger P, Kinsner-Ovaskainen A, Pallas M, Rimbau V, Rodriguez-Farre E, Sunol C, Vericat JA, Zurich MG (2009) Neuronal in vitro models for the estimation of acute systemic toxicity. *Toxicol in Vitro* 23, 1564–69. [PubMed: 19615435]

Recommended Reading

- Sambrook J, and Russell D (2001) *Molecular Cloning: A Laboratory Manual* (Hardcover). Third Edition, Cold Spring Harbor Laboratory Press.
- Primrose SB and Twyman RM (2007) *Principles of gene manipulation and Genomics*. Seventh Edition, Blackwell Publishing.
- Glick BR, and Pasternak JJ (2003) *Molecular Biotechnology. Principles and Applications of Recombinant DNA*. Third Edition, ASM Press.
- Brown TA (2008) *Gene Cloning and DNA analysis*. Fifty Edition Blackwell Publishing.
- Chen BY, and Janes HW (2002) *PCR cloning protocols*, Second Edition, Humana Press.

Figure 1: pCIG overexpression vector map.

Main features and restriction sites are shown. Unique restriction sites useful for cloning are shown in *italics*. MCS: multiple cloning site; Amp^R: gene encoding ampicillin resistance.

Figure 2: Overview of the reaction catalyzed by DNA ligase.

Firstly, a complex between the enzyme and the cofactor is formed, in a process in which ATP is hydrolyzed into AMP, and the complex binds the 5'-phosphate end of the nick. In a second step, the 3'-OH end of the nick attacks the AMP-DNA bond, releasing AMP and forming a new covalent bond in the phosphodiester chain.

Figure 3: Insert-vector ligation and strategies to avoid recircularization.

A) Ligation reaction of an EcoRI cleaved DNA fragment (red) in an EcoRI cleaved vector (black) without further treatment. Self-circularization of vector is highly favored. **B)** Second cleavage of vector and insert with XhoI make incompatible both ends of the vector, avoiding self-circularization. **C)** Vector dephosphorylation with alkaline phosphatase eliminates 5'phosphate groups at the ends of the vector, necessary for recircularization. Note that in **B)**, as well as in **C)**, the only possible ligation reaction is between vector and insert.

Figure 4: Steps of the first cycle of a PCR (denaturation, annealing of primers and primer extension).

Target region to be amplified is shown in red. Red arrows represent chemically synthesized oligonucleotides designed to anneal at the flanks of the amplicon. Dashed lines represent newly synthesized DNA strand.

Figure 5: First three cycles of a PCR and exponential amplification of the amplicon.

Target region and oligonucleotides are shown as in Figure 4. At the second cycle of amplification, two DNA molecules that comprise exactly the amplicon are generated (boxed in red). Progression of PCR amplifies these molecules exponentially.

Figure 6: PCR-based site-directed mutagenesis.

Region to be mutated in the template is shown in red. Mutation point in the primers is shown as a zigzag line. Two independent PCRs (PCR 1 and PCR 2) generate two overlapping fragments of the amplicon that have incorporated the mutation (strand 1–1' and strand 2–2'). Mixing of this fragments and a single round of polymerase reaction generate a template that incorporates the mutation and can be amplified in a third round of PCR (PCR 3).

DOTMA

DOGS

DC-Cholesterol

DOPE

Figure 7: Chemical structures of some lipids used in lipofection.

DOTMA (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride); DOGS (Spermine-5-carboxy-glycine dioctadecyl-amide); DC-Cholesterol (3β[*N*-(*N',N'*-dimethylaminoethane) carbamoyl]-cholesterol); DOPE (L-dioleoyl phosphatidylethanolamine).

Figure 8. Transfection of mouse cerebellar granule cells by electroporation.

Mouse cerebellar granule cells were transfected by electroporation (microporation) with 5 μ g of a plasmid encoding the EGFP. 48 hours post microporation, the cells were analyzed by phase contrast (A) and fluorescence microscopy (B).

Table 1:
Recommended conditions to perform a PCR reaction.

Fw = forward primer; Rv = reverse primer.

PCR MIX (50 μ L of final volume)			THERMAL PROGRAM	
	Stock concentration	Final concentration		
5 μ L Buffer	(10x) (with 18 mM MgCl ₂)	1x 1.8 mM	1	94°C / 5'
1 μ L dNTPs	(10 mM each)	200 μ M	2	94°C / 30"
2.5 μ L DMSO	(100%)	5%	3	(tm-2°C) / 30"
1 μ L Fw primer	(10 μ M)	200 nM	4	72°C / (1 min/Kb)- go to step 2, 34 times-
1 μ L Rv primer	(10 μ M)	200 nM	5	72°C / 10'
2 μ L cDNA	(50 ng/ μ L)	2 ng/ μ L		
0.5 μ L <i>Taq</i> DNA pol (High Fidelity)	(5 U/ μ L)	50 mU/ μ L		
37 μ L H ₂ O				