Chapter 11

Manipulation and Study of Gene Expression in Neurotoxin-Treated Neuronal PC12 and SH-SY5Y Cells for *In Vitro* Studies of Parkinson's Disease

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Abstract

Neuronal PC12 and SH-SY5Y cells are highly suitable *in vitro* models for study of the neurodegenerative mechanisms occurring in Parkinson's disease (PD). Differentiated PC12 and SH-SY5Y cells bear many similarities to the neuronal populations affected in PD, and they provide a convenient source of large amounts of homogeneous material for biochemical and molecular downstream applications. In the present review, we describe how to differentiate PC12 and SH-SY5Y cells into neuron-like cells and provide protocols for their transfection with plasmids and infection with viral particles to manipulate gene expression. We also describe how to treat neuronal PC12 and SH-SY5Y cells with the classical PD neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-pyridinium ion (MPP+). Finally, we give detailed methods for several downstream applications useful for the analysis of cell death pathways in PD.

Keywords: Parkinson's disease, PC12, SH-SY5Y, differentiation, 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium (MPP+), transfection, lentiviral infection, survival assay, immunofluorescence, quantitative polymerase chain reaction, Western immunoblotting

1. Introduction

The etiology of Parkinson's disease (PD) is still unknown and likely due to combinations of environmental and genetic factors, ultimately leading to the degeneration of various neuronal populations [1–4]. Neuron death in models of PD requires the transcription-dependent induction of specific pro-death genes. Identifying and manipulating transcriptionally regulated genes that mediate neuron degeneration in PD is a promising strategy for treating PD without knowing

the proximal initiating causes of the disease [5]. Tumor-derived cell lines expressing neuronal properties such as PC12 and SH-SY5Y cells are highly suitable *in vitro* models to apply this strategy. PC12 is a cell line initially isolated from a pheochromocytoma of the rat adrenal medulla [6]. Undifferentiated PC12 cells exit the cell cycle and differentiate into neurons after 1 week of exposure to nerve growth factor (NGF). SH-SY5Y is a human neuroblastoma cell line originally derived from a metastatic bone tumor biopsy. Neuroblast-like SH-SY5Y cells can be withdrawn from the cell cycle and differentiated into a more mature neuron-like phenotype by sequential exposure to retinoic acid and brain-derived neurotrophic factor (BDNF) [7]. Differentiated PC12 and SH-SY5Y cells extend long neurite-like processes, express neuron-specific markers and synthesize the catecholamine neurotransmitters dopamine and norepinephrine. PC12 and SH-SY5Y neurons highly resemble some of the neuronal populations affected in PD, such as the dopaminergic neurons of the substantia nigra *pars compacta* and the noradrenergic neurons of the locus coeruleus and peripheral sympathetic ganglia [1–4]. Under appropriate conditions, neuronal PC12 and SH-SY5Y cells are dependent on the trophic actions of NGF and BDNF, respectively [6, 7]. They are also sensitive to the classical PD neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-pyridinium ion (MPP+). Therefore, differentiated PC12 and SH-SY5Y cells have been widely used as models to study PD neurodegenerative mechanisms [8–13]. They are easily transfected or infected with viral particles to manipulate gene expression. They additionally provide a convenient source of large amount of homogeneous material for biochemical and molecular downstream applications and the analysis of cell death pathways. These properties also make these lines very useful for drug screening studies.

In this chapter, we will introduce how to apply 6-OHDA and MPP+ to differentiated PC12 and SH-SY5Y cells *in vitro*. Then, we will describe two ways to manipulate gene expression: plasmid transfection and lentiviral-mediated plasmid delivery in cellular PD models. In addition, we will discuss different methods to assess cell survival.

2. Materials

2.1. Coating plasticware

1. For PC12 cells: Reconstitute 10 mg of lyophilized rat tail collagen (Roche) with 5 mL of 0.2% acetic acid. Working dilution is 1:20 in sterile double distilled or deionized water (ddH_2O).
2. For SH-SY5Y cells: Matrigel (BD) should be thawed on ice overnight. Swirl vial gently and make 200 μL aliquots in microcentrifuge tubes using a pre-cooled pipet. Aliquots can be stored at −20°C.

2.2. Cell medium

2.2.1. PC12 cell medium

1. Complete growth medium: RPMI 1640 cell culture medium supplemented with 10% heat inactivated horse serum (Sigma), 5% fetal bovine serum (FBS) and the antibiotics penicillin/streptomycin (pen/strep) (50 units/50 μg/mL final concentration).

2. Differentiation medium: RPMI 1640 cell culture medium supplemented with 1% heat-inactivated horse serum and pen/strep (50 units/50 μg/mL final concentration). NGF should be added to the differentiation medium for PC12 cell neuronal differentiation. Recombinant human or murine NGF stock concentration is 50 μg/mL and should be diluted 1000-fold directly into the culture medium. Medium with diluted NGF should not be stored and should be freshly prepared.
3. Freezing medium: Complete medium containing 10% dimethyl sulfoxide (DMSO).

2.2.2. SH-SY5Y cell medium

1. Growth medium: DMEM supplemented with 10% heat inactivated FBS (Gemini Bioproducts), 2-mM L-glutamine and antibiotics pen/strep (50 units/50 μg/mL final concentration).
2. Differentiation medium I: DMEM supplemented with 5% heat inactivated FBS (Gemini Bioproducts), 2 mM L-glutamine and antibiotics pen/strep (50 units/50 μg/mL final concentration). Retinoic acid is added freshly before use from a 0.5 mM stock (50 ×) in sterile ddH_2O.
3. Differentiation medium II:
   a. Neurobasal medium
   b. B27 supplement (50×)
   c. Antibiotics pen/strep (50 units/50 μg/mL final concentration).
   d. GlutaMAX (Life Technologies) supplied as a 200 mM (100×) liquid stock.
   e. 0.25 M (125×) dibutyryl cyclic adenosine monophosphate (dibutyryl cAMP) in sterile phosphate-buffered saline (PBS).
   f. 100 mM (2000×) stock solution of recombinant human brain-derived neurotrophic factor (rhBDNF) in sterile ddH_2O. Store at −20°C. Avoid multiple freeze and thaw cycles.
   g. 2 M (100×) stock solution of KCl in sterile ddH_2O.

Reagents a–c can be added to Neurobasal medium in advance and kept at 4°C. Reagents d-g should be added to Neurobasal medium with serum just before use.

4. Trypsin: 0.05% trypsin solution (Gibco).
5. Freezing medium: Growth medium containing 10% DMSO.

2.2.3. HEK293T cell medium

1. DMEM cell culture medium supplemented with 10% FBS (Gemini Bioproducts).
2. 0.05% Trypsin-EDTA (Gibco).

2.3. Parkinson's disease toxins

Just before the treatment, prepare a 10 mM 6-OHDA (Tocris) or a 100 mM MPP+ (Sigma) stock solution in sterile ddH_2O.

2.4. Transfection and immunostaining reagents

1. Lipofectamine 2000 (Invitrogen).
2. 1 mg/mL Hoechst 33342 dye (Molecular Probes).
3. 4% formaldehyde: Dilute 16% (4×) formaldehyde aqueous solution (EMS) in PBS before use.

2.5. Lentiviral vector production

1. Calcium phosphate transfection buffer: Prepare and sterile-filter a 250 mM $CaCl_2$ solution in ddH_2O. Sterile-filter a 2 × HBSS (Hank's balanced salt solution) containing 50 mM HEPES, 280 mM NaCl and 15 mM Na_2HPO_4 in ddH_2O. The pH of the 2 × HBSS solution should be precisely adjusted to 7.03–7.04 with 1 M NaOH.

2.6. Western blot

1. 1 × cell lysis buffer: In a 15-mL conical tube, add 1 mL of 10 × cell lysis buffer (Cell Signaling), one tablet of complete mini EDTA-free protease inhibitor cocktail (Roche) and 9 mL of ddH_2O. Mix for 30 min by rotation, prepare 1 mL aliquots and freeze at −20°C.
2. Protein sample buffer: 4 × NupageLDS sample buffer (Life Technologies), 10 × (500 mM) dithiothreitol (Nupage Reducing Agent, Life Technologies) and ddH_2O to achieve a 1–2 μg/μL final protein concentration.
3. 1 × running buffer (1 L): 50 mL of 20 × MOPS running buffer (Life Technologies) and 950 mL of ddH_2O.
4. 1 × transfer buffer (1 L): 50 mL of 20 × transfer buffer (Life Technologies), 150 mL of 95% ethanol and 800 mL of ddH_2O.
5. Ponceau S: 0.1% w/v Ponceau and 5% acetic acid in ddH_2O.
6. Washing buffer: 1 × TBS (Tris-buffered saline) + 0.1% Tween-20 (TBST).
7. Blocking solution: TBST + 5% dry milk. Store at 4°C.

2.7. Survival assay

1. 10 × cell lysis counting buffer (100 mL): cetyldimethyl-ethanolammonium bromide (5 g), NaCl (0.165 g), glacial acetic acid (2.8 mL), 10% Triton X-100 (50 mL), 1 M $MgCl_2$ (2 mL), 10 × PBS (10 mL) and ddH_2O (35.2 mL). The working dilution is 1 × in distilled water.

3. Methods

3.1. PC12 cells

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. It has been found that PC12 cells stop dividing and differentiate to a neuronal phenotype after treatment with NGF [6]. PC12 cells are a good model for studying neuron differentiation and degenerative disease.

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3.1.1. Coating plasticware

Dilute 2 mg/ml stock collagen solution in sterile water into a 0.1 mg/ml working collagen solution as described in section 2.1 and ensure the solution covers the entire surface of the dish. Use 1 mL of the diluted collagen solution per 10-cm cell culture dish. The volume should be adjusted accordingly for different dish sizes. Incubate the coated dishes for 4–6 h at room temperature and remove the excess and air-dry in a cell culture hood. Alternatively, do not remove the excess and allow the culture dishes to dry in the cell-culture hood at room temperature overnight.[1]

3.1.2. Maintaining PC12 cells

1. Seed undifferentiated PC12 cells in 5–8 mL of complete growth medium in a collagen-coated 10-cm dish and split them, when they reach 80–90% confluence.
2. When cells reach confluence, aspirate ¾ of the medium from the plate.
3. Add 2 mL of fresh complete medium and pipet up and down over the cell monolayer to detach the cells and break up cell clumps.
4. Triturate the cells several times with the pipette to further break up cell clumps.
5. Split the cells into 2–3 10-cm dishes and add complete medium to a final volume of 5–8 mL.
6. Change the complete medium every 2 days by removing ¾ of the old medium and gently adding fresh complete medium up to 5–8 mL, leaving the cell monolayer undisturbed.

3.1.3. Differentiating PC12 cells

1. Detach PC12 cells from the plate as described above with complete medium.
2. Dilute the cell suspension with the differentiation medium and add NGF to a final concentration of 50 ng/mL. One 10-cm confluent plate of PC12 cells can be used to generate 5–20 cultures of the same size for differentiation. The dilution of the cell suspension should be adjusted according to specific experimental requirements.[2]
3. Plate the cells on the appropriate collagen-coated dish.
4. Change the differentiation medium every 2–3 days (using fresh NGF) for up to 7–10 days.

After 24 h, cells begin to extend neurites. 7–10 days of differentiation is necessary before proceeding to toxin treatment.

[1]Once coated, these dishes can be kept for 2–3 weeks. Because PC12 cells tend to detach from the plasticware easily, the coated plasticware should not be used beyond 3 weeks.
[2]Usually, naïve PC12 cells are grown in 10-cm dishes. When using differentiated PC12 cells, different type of plates can be used to satisfy experimental requirements. For example, 24 or 48 well plates can be used for viability assays. 6-well or 12-well plates can be used for mRNA purification and protein extracts.

3.1.4. Freezing PC12 cells for storage

1. Detach the cells from the plates using complete medium and place them in 15 mL conical tubes.
2. Centrifuge the cell suspension at 1000 rpm for 5 min and discard the supernatant.
3. Add 2 mL of complete medium containing 10% DMSO and mix them gently.
4. Place the cell suspension in cryotubes.
5. Place the cryotubes in an isopropanol-freezing container.
6. Keep the container at −80°C overnight and transfer the tubes into liquid nitrogen for long-term storage. The cryotubes can be stored at −80°C for up to several months. For longer periods of storage, the cells must be kept in liquid nitrogen.

3.2. SH-SY5Y cells

For SH-SY5Y cell differentiation, several protocols are commonly used. Here, we will introduce an easy and reproducible procedure to generate a fully differentiated homogeneous population of neuron-like cells.

3.2.1. Coating plasticware

1. Thaw BD Matrigel in a vial covered by foil on ice overnight, and swirl it to ensure that it is evenly mixed.
2. Dilute Matrigel using pre-cooled pipets in cold DMEM (1:10).
3. Add enough Matrigel solution to cover the entire cell-culture dish surface.
4. Incubate at room temperature for at least 1 h.
5. Rinse with serum-free DMEM two times before use.

3.2.2. Plating cells

1. If SH-SY5Y cells are received or stored in a frozen state (−80°C or in liquid nitrogen), thaw the vial at 37°C in a water bath and plate them as soon as possible to limit exposure to the toxicity of DMSO.
2. Prepare 10 mL of warm (37°C) growth medium in a 15-mL Falcon tube.
3. Remove the thawed cell suspension from the cryotube and place it into the pre-warmed complete medium.
4. Carefully mix the cell suspension and centrifuge for 3 min at 1000 rpm at room temperature.

5. Discard the supernatant and add 2 mL of fresh growth medium and pipet up and down to resuspend the cell pellet.
6. Place the cells into the Matrigel-coated plate and add another 8 mL of growth medium (for a 10-cm plate). Gently swirl the plate back and forth and left to right to prevent the cells from concentrating at the center of the plate.

3.2.3. Maintaining SH-SY5Y cells

Once the cells are plated, passage them at every 2–3 days at 1:3 to 1:4 ratio (it is recommended to subculture the cells when they reach 70–90% confluence), depending on your experimental requirement.

1. Aspirate the entire medium from the 15-cm plate.
2. Wash the cells with 3–8 mL of warm PBS (37°C).
3. Add 1–2 mL of trypsin. Swirl the plate to cover the entire cell surface with trypsin solution and for 4–5 min.
4. Use hands to tap the bottom of the dish. At this point, the cells will detach from the plate.
5. After applying the medium onto the entire surface, triturate the cells several times with the pipette to break down cell clumps.
6. Split cells at different ratios as experimentally required into Matrigel-coated plates.
7. Bring the total volume per plate up to 6–9 mL.

3.2.4. Differentiation of SH-SY5Y cells

1. Remove growth medium and wash the cells with warm PBS in a 15-cm plate.[3]
2. Add 2 mL of trypsin and incubate for 2–3 min at room temperature.
3. Dilute the cell suspension in growth medium and triturate it to get a homogenous cell suspension prior to transfer to a new flask.[4]
4. Seed 2–3 × 10^3 cells/cm^2 cells into Matrigel-coated plates and let them sit overnight in the cell culture incubator so that the cells attach to the plates.
5. The next day, remove the growth medium and add the differentiation medium I (DMEM, 5% FBS, 2 mM L-glutamine and pen/strep) supplemented with retinoic acid at a final concentration of 10 μM.

[3]The passage number of SH-SY5Y cells required for differentiation should be as low as possible. If cells switch to a fibroblast-like phenotype after several passages, they should not be used for differentiation.
[4]Excessive dilution (more than 1:4) and splitting of SH-SY5Y cells before they reach confluence will block the cells from achieving differentiation.

6. Change the culture medium every 2 days and culture the cells under these conditions for 5 days.
7. Subsequently, remove the medium and replace it with differentiation medium II (Neurobasal medium, 5% B-27, 2 mM GlutaMAX, 2 mM dibutyryl-cAMP, 20 mM KCl, 50 ng/mL rhBDNF and pen/strep).
8. Keep the cells in differentiation medium II for an additional 5 days before using them.

3.2.5. Conserving SH-SY5Y cells

1. Wash SH-SY5Y cells with PBS.
2. Add 2–4 mL of 0.05% trypsin for 2–3 min at room temperature.
3. Detach the cells from the plate with 2 mL of growth medium.
4. Centrifuge the cell suspension at 1000 rpm for 5 min and remove the supernatant.
5. Add 2 mL of growth medium containing 10% DMSO and mix them gently.
6. Place the cell suspension in cryotubes.
7. Place the cryotubes in an isopropanol-freezing container and place it at −80°C overnight. Transfer the cryotubes into liquid nitrogen for long-term storage.

3.3. Parkinson's disease cellular models

Addition of 6-OHDA or MPP+ to NGF-differentiated PC12 and SH-SY5Y cells is used as PD cellular models.

1. Replace the medium with fresh medium before any treatment.
2. Prepare stock solutions of the toxin before use.
3. Since 6-OHDA and MPP+ are light sensitive and unstable, their solutions should be wrapped in aluminum foil to avoid light and prepared just before each use. Minimize exposure to light and air.
4. To treat neuronal PC12 cells[5]:
   a. 6-OHDA:

Measure 6-OHDA into a small tube covered with foil. Prepare a 10 mM stock in sterile ddH_2O. Filter-sterilize using a 10 mL syringe and a 0.2 μm filter. Use 6-OHDA at a 50–100 μM

[5]Both 6-OHDA and MPP+ are usually used as toxins to mimic PD. Dopamine transporters will specifically take up these toxins. Therefore, the density of the dopamine transporter on cells and the total number of cells determine the toxicity range. To get a certain amount of cell death, the cell density and the toxin concentrations should be determined empirically. The concentrations listed above are intended to cause 40–60% cell death. For initial toxin treatment experiments, viability should be monitored as recommended at 24 and 48 h.

final concentration. Take appropriate caution at all times to keep from coming into contact with the 6-OHDA powder or solution.

b. MPP+:

Prepare a 100-mM MPP+ stock solution in a tube covered with foil with ddH_2O and sterile-filter using a 0.2-μm filter. Use MPP+ at a 500 μM to 1 mM working concentration. Take appropriate caution at all times to keep from coming into contact with the MPP+ powder or solution.

5. To treat differentiated SH-SY5Y cells (see footnote 5):

Prepare toxin stocks as above, but the final concentration of 6-OHDA should be much lower: 10–20 μM for 6-OHDA. The final concentration of MPP+, however, should be between 1 and 3 mM. These concentrations of toxins will result in about 50% cell death.

6. For small wells (24- or 48-well plates), because small volumes are more prone to pipetting errors, use a diluted stock of 6-OHDA. Dilute the 10 mM stock at 1:10 in medium to make a 1 mM stock before use.

7. After treatments, assess the experiment at the desired time.

3.3.1. Manipulation of gene expression in cultured cells to study PD

To study gene function in PD cellular models, gene overexpression and silencing are powerful tools. Here, we take PC12 cells as an example to introduce two ways to manipulate gene expression: plasmid transfection and lentiviral-mediated plasmid delivery. We will also introduce several methods to assay PC12 cell survival in these two systems.

3.4. Manipulation of gene expression by transfection

3.4.1. Transfection

Neuronal PC12 cells are very difficult to transfect with plasmids (less than 5% transfection rate), but transfection is still a good way to study some gene functions following PD toxin treatments. The low transfection rate makes it easy to observe individual cell phenotype changes, and transfection is also easier to handle than virus infection. Usually the plasmids used for transfection contain fluorescent markers such as GFP, which makes it possible to observe the transfected population. Transfection is also useful for gene regulation studies using luminescent reporters such as luciferase.

1. Seed PC12 cells as mentioned above.

2. Add NGF to differentiate PC12 cells for at least 3–4 days.

3. Transfect neuronal PC12 cells with plasmids (usually with some fluorescent marker, such as green fluorescent protein (GFP)) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

4. After 48 h, observe transfected cells (as judged by expression of the fluorophore) with a fluorescence microscope. If sufficient cells are transfected, the cultures can be treated with 6-OHDA or MPP+ as experiments require.
5. At various times after treatment with the toxins, viable transfected neuronal PC12 cells can be identified and target genes in the transfected cells can be studied by co-immunostaining.

3.4.2. Viability assay for transfected cells

Cell number counting or apoptotic nuclei assessment can be used to assess cell survival.[6]

a. Strip counting or whole-well counting: the number of healthy, GFP positive cells is counted in a field consisting of a strip across the diameter of each well or the whole well. The survival rate is calculated and normalized to the number of cells counted in the control wells. The number of transfected cells counted should be at least 400.[7]

b. Apoptotic nuclei assessment: Hoechst dye 33342 is applied to stain nuclei either of living cells or after fixation with formaldehyde.

For living cells:

1. 1 mg/mL Hoechst 33342 is added the medium to a final concentration of 1 μg/mL for 5 min.
2. Remove the culture medium and replace with fresh medium that does not contain Hoechst 33342.

For fixed cells:

1. Remove the medium.
2. Add 4% formaldehyde for 10 min, wash 3 times with PBS.
3. Stain with Hoechst 33342 (at 1 μg/mL final concentration) for 5 min.
4. Replace with PBS without dye.

The cells that possess both GFP and condensed nuclei/fragmented chromatin are scored as apoptotic. The number of apoptotic cells is calculated relative to the number of GFP+ cells in the same well (see footnote 7).

3.4.3. Immunostaining after transfection

1. 24 or 48 h after transfection, fix the cells with 4% formaldehyde for 10 min.
2. Permeabilize cells with 0.5% Triton X-100 for 10 min at room temperature.

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[6]All the results should be repeated as least three times, and analysis by Student's test or ANOVA with Tukey's *post hoc* test.

[7]If the fluorescence signal is weak, immunostaining will enhance the signal. GFP antibody can be used enhance the signals. After that, assess cell viability through cell survival assessments as described.

3. Block cells with 5% bovine serum albumin (BSA) in PBS for 40 min.
4. Stain PC12 cells with specific primary antibody at appropriate dilution rate in 5% BSA overnight at 4°C.
5. Wash cells 3 times with PBS, 10 min each time.
6. Incubate cells with secondary antibody for 1 h at room temperature.
7. Dilute Hoechst 33342 dye to 1 μg/mL in PBS.
8. Stain cells for another 5 min.
9. Wash cells with PBS.

Cells are observed and scored under a fluorescence microscope. An example of transfected and immunostained neuronal PC12 cell culture is shown in **Figure 1**.

3.5. Manipulation of gene expression by lentiviral vector infection

Lentiviral vectors derived from Human immunodeficiency virus type 1 are able to infect differentiated neurons and stably integrate into the host genome, resulting in long-term expression of the transgene. A major advantage of lentiviral-mediated gene delivery, compared to the transfection procedure described above, is that it will typically achieve a 80–90% transduction rate in a PC12 or SH-SY5Y neuronal cell population. This high transduction rate is crucial for the use of downstream applications requiring highly efficient manipulation of gene expression, such as qPCR or Western immunoblotting.

The strategy to produce replication-defective lentiviral particles has been to remove all dispensable genes from the HIV-1 genome and separate the cis-acting sequences from the trans-acting elements required for viral particle production, infection and integration [14–16]. The

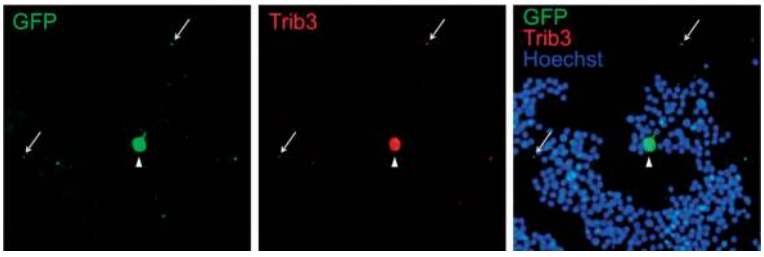

Figure 1. Transfection of the pro-apoptotic Tribbles pseudokinase 3 (Trib3) in neuronal PC12 cells. PC12 cells were differentiated into neurons with NGF for 3 days and transfected with a construct co-expressing full length Trib3 and GFP using Lipofectamine 2000. After 48 h, cells were fixed with 4% PFA and immunostained for GFP (chicken anti-GFP primary antibody and goat anti-chicken Alexa fluor 488 secondary antibody, Life Technologies), Trib3 (rabbit anti-Trib3 primary antibody, Calbiochem, and goat anti-rabbit Alexa fluor 568 secondary antibody, Life Technologies) and nuclei were stained with Hoechst 33342. Under basal conditions, neuronal PC12 cells express undetectable levels of endogenous Trib3. A successfully transfected neuronal PC12 cell is shown here co-expressing GFP (left) and Trib3 (center) in the cell body (arrowhead) and in discrete puncta in the processes (arrows). A merged image with additional Hoechst staining of the nuclei is shown on the right.

third-generation lentiviral vector system contains four plasmids: an expression vector containing the cis-acting sequences and three additional packaging plasmids: pMDL, pRev and pVSVG, providing the trans-acting factors. pMDL encodes a gag-pol precursor that is processed into an integrase and a reverse-transcriptase, as well as structural capsid proteins. Rev interacts with RRE, a cis-acting element enhancing the nuclear export of viral mRNAs. pVSVG encodes an envelope glycoprotein that confers the viral particle the ability to transduce a broad range of cell types. The second-generation lentiviral vector system contains only three plasmids: an expression vector containing the cis-acting sequences including Rev and two additional packaging plasmids: psPAX2 and pVSVG, providing the other trans-acting factors.

Manipulation of gene expression by lentiviral vector infection is a multistep process requiring, first, design and cloning of lentiviral constructs; then transfection of these constructs into packaging cells (HEK 293 cells); followed by collection, purification and concentration of the lentiviral particles; and finally, infection of neuronal cells. Design and cloning of lentiviral vectors will not be discussed in this methodological review, please refer to Tiscornia et al. [17] for a detailed protocol.

3.5.1. HEK 293T cells growth and maintenance

Grow and propagate HEK293T cells in DMEM +10% FBS in 15-cm dishes. Split the cells when they reach 80–90% confluence.[8]

1. Aspirate the medium of a confluent plate of HEK293T cells.
2. Add 5 mL of 1 × trypsin-EDTA. Swirl the plate to cover the entire cell surface with trypsin solution and place the plate in a 37°C incubator for 1 min.
3. Add 10 mL of DMEM +10% FBS to dilute and inactivate the trypsin solution and pipet up and down over the cell monolayer to detach the cells and break down cell clumps.
4. Transfer 5 mL of the cell suspension in a new 15-cm dish with 13 mL of fresh DMEM +10% FBS. Swirl the plate to evenly spread the cells.

3.5.2. Seeding HEK293T cells for lentivirus production (Day 1)

This protocol describes the production of two batches of virus (i.e., four 15-cm dishes total: two 15-cm dishes are needed for each batch of lentivirus), as it is usually needed for a given experiment: one batch (two 15-cm dishes) of lentivirus containing an expression vector designed to manipulate gene expression and one batch (two 15-cm dishes) of lentivirus containing a control expression vector. The lentiviral vectors usually contain fluorescent markers such as GFP.

[8]HEK293T cells should not be allowed to become more than 80–90% confluent during maintenance. To ensure high transfection efficiency, HEK293T cells should be of low passage number and should demonstrate rapid growth when seeded for lentivirus production.

1. Resuspend HEK293T cells from 80 to 90% confluent 15-cm dishes with trypsin-EDTA and DMEM +10% FBS, as described above.
2. Count the cells concentrated in the trypsinized cell suspension with a hemacytometer.
3. Seed about 2 × 10^7 HEK293T cells per 15-cm plate. Adjust the total volume to 18 mL/plate with DMEM +10% FBS and swirl the plate to evenly spread the cells.

3.5.3. Calcium phosphate transfection of the lentiviral plasmids (Day 2)

1. Make sure that the cells seeded the day before are healthy and close to 80–90% confluent: they should still have room to undergo 1–2 cell divisions.
2. For each batch of virus, prepare the corresponding plasmid mix in a 15-mL tube containing 4 mL of 250 mM $CaCl_2$. For second-generation lentiviral system, use 30 μg of psPAX2, 20 μg of VSVG and 40 μg of expression vector. For third-generation lentiviral system, use 20 μg of CMV-VSVG, 20 μg of pMDLg/pRRE, 20 μg of RSV/REV and 40 μg of expression vector.
3. Prepare two separate 15-mL tubes, each containing 4 mL of 2× HBSS solution. To ensure high transfection efficiency, the pH of the HBSS solution should be precisely adjusted to 7.03–7.04 with NaOH.
4. Drop by drop, and by continuously vortexing, add the 4 mL of the $CaCl_2$ + plasmid mix solution to the 4 mL of HBSS solution, for each virus batch. Avoid the formation of precipitates. You now have 8 mL of transfection mix ready for each virus batch.
5. Add 4 mL of the transfection mix to each 15-cm dish, swirl the plates gently and place them back into the incubator.
6. After 4–5 h, replace the medium of the transfected cells with 18 mL of fresh DMEM +10% FBS.

3.5.4. Collection of the lentiviral particles (Day 4 and Day 5)

From this point on, viral particles accumulate in the supernatant. Proceed with appropriate precautions when manipulating the supernatants.[9]

1. On Day 4, observe the cells and check the transfection efficiency. Cells should be reaching confluency and, if a marker (such as GFP) is present in the expression vectors, transfection may be assessed visually. The transfection efficiency should be >90%.
2. Harvest the first supernatant by pooling 18 mL from each plate of the same virus batch in a 50-mL conical tube. Seal the tubes with Parafilm and place them on a designated rack at 4°C.

[9]Perform all manipulations in a biosafety cell culture cabinet. When working in the cabinet, wear two pair of gloves at all times and remove the second pair when leaving the area. All containers should be sealed with Parafilm (or placed in a second vessel) when transported outside of the biosafety cabinet. All the material and reagents should be decontaminated in 10% bleach. The biosafety cabinet (containing all the contaminated material and reagents) should be placed under UV light for at least 30 min at the end of each procedure involving contaminated material and reagents.

3. Replace the supernatant with 18 mL of fresh DMEM +10% FBS.
4. On Day 5, harvest the second supernatant, following the same procedure.

3.5.5. Purification of the lentiviral particles

1. Spin the 50-mL conical tubes containing the supernatants for 5 min at 1000 g to pellet the cellular debris.
2. Pool and filter the first and the second supernatants from the same virus batch in 0.45 μM 115 mL filter units. Then proceed to concentrate the virus particles as below.

3.5.6. Concentration of the lentiviral particles

Several methods can be used to concentrate the virus: Ultracentrifugation, the most well-described method [18], filtration with a centrifugal filter unit equipped with a 100 kDa molecular weight cut-off membrane (Millipore cat. No. UFC910024) and regular centrifugation with Lenti-X concentrator. The Lenti-X method gives the same yield as the other methods and is, in our hands, the quickest and the most convenient. Therefore, we will describe this method here.

1. Re-distribute the filtered viral supernatant in a 50-mL conical tube (36 mL/tube) and add 1:3 of the supernatant volume of Lenti-X concentrator (12 mL/tube).
2. Seal the tubes with Parafilm and mix by gentle inversion.
3. Incubate the supernatant + Lenti-X mix for 2 h up to 72 h at 4°C.
4. Centrifuge at 1500 g for 45 min at 4°C. An off-white pellet will become visible.
5. Discard the supernatant and resuspend the pellet with 200 μL of PBS (no $CaCl_2$, no $MgCl_2$).
6. Store the concentrated viral particles in single-use aliquots at −80°C. Avoid freeze–thaw cycles.

3.5.7. Titration of the lentiviral particles

Depending on your experimental needs, a precise determination of the biological titer of the purified lentiviral vectors might be necessary and conducted as follows.

1. The day before titration, seed 10^5 HEK293T cells in each well of a 24-well plate.
2. On the day of titration, prepare 6 tubes, each containing 45 μL of DMEM +10% FBS.
3. Add 5 μL of viral preparation in the first tube and mix.
4. Make a 10-fold serial dilution, by pipetting 5 μL of the diluted virus into the next tube, and so forth.
5. Add 450 μL of DMEM +10% FBS in each tube.

6. Aspirate the entire medium of each HEK293T cell-culture well and add 500 μL of the lentivirus dilutions.
7. 48 h after infection, count the total number of GFP+ cells in a well that shows a number of GFP+ cells between 50 and 500. If the signal is too weak, perform an immunofluorescence procedure with an anti-GFP primary antibody.
8. The biological titer (BT) of the virus preparation corresponds to the number of GFP+ cells counted in a given well (N) divided by the dilution factor (DF) of the same well multiplied by 5. BT = (N/5*DF).

3.5.8. Lentiviral infection of neuronal PC12 cells

1. Seed PC12 cells as described above.
2. Add NGF to differentiate PC12 cells for at least 3–4 days.
3. On the day of lentiviral infection, replace the medium with fresh differentiation medium.
4. Add concentrated viral particles (1×10^7 viral particles/cm^2 of culture area[10]) and do not change the medium for 24 h.
5. Maintain the infected cells by changing the differentiation medium every 2–3 days, as usual.
6. After 3–7 days, observe the infected cells. Check the transduction efficiency by evaluating the expression of the fluorescent marker (such as GFP) under a fluorescence microscope. The transduction efficiency should be 80–90%. If enough cells are infected, the cultures are ready to be treated with 6-OHDA or MPP+ and used for downstream applications.

3.6. Downstream application: qPCR

The amount of an expressed gene in a cell can be measured by the number of copies of the corresponding mRNA transcript present in a given sample. In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction (PCR) is the most common method for amplifying DNA. For mRNA-based PCR, the extracted mRNA is converted to cDNA by reverse transcription.

3.6.1. Harvesting cells for qPCR

1. Take the cells out of the incubator and place them on ice.
2. Remove the medium gently and add TRI reagent (Molecular Research Center) in each well (100 μL of TRI reagent/cm^2 of culture dish area). Refer to the manufacturer's protocol and handling notes for additional information on TRI reagent.[11]

[10]The amount of concentrated virus vector to use is mostly dependent on cell density in your culture dish and should be determined empirically. Typically, when infecting moderately sparse neuronal PC12 cultures (avoid clumps at all cost), we use 0.1 up to 5× 10 viral particles/cm of culture area, and usually get a 80% infection rate. If the viral preparation is good but your infection rate is low, consider decreasing the density of your cell culture.
[11]TRI reagent contains harmful compounds such as phenol and guanidine thiocyanate. Manipulate with caution under a chemical hood and wear gloves at all times.

3. Use a cell lifter to detach the cells from the bottom of the well and homogenize the cell lysate.
4. Pipette the cell lysate into labeled microcentrifuge tubes and pipette up and down 2–3 more times to further homogenize the cell lysate. At this point store the homogenates at −80°C before proceeding to RNA extraction.

3.6.2. RNA extraction

1. Take the samples out of the −80°C freezer and allow them to thaw at room temperature for 10 min.
2. Add 10 μL of bromochloropropane/100 μL of TRI reagent.
3. Cap the tubes tightly and vortex vigorously for 15 s.
4. Incubate the mixture at room temperature for 10 min.
5. Centrifuge at 12000 g for 10–15 min at 4°C. Meanwhile, label a new set of microcentrifuge tubes.
6. Transfer the aqueous phase (top layer) to a new tube. RNA remains in the aqueous phase whereas DNA is in the interphase and proteins remain in the organic phase.
7. Add 50 μL of isopropanol/100 μL of TRI reagent solution.
8. Vortex for 10 s.
9. Incubate at room temperature for 10 min.
10. Centrifuge at 12000 g for 8 min at 4–25°C. At this point, a translucent (gel-like) to white pellet should become visible at the bottom of the tube.
11. Carefully remove the supernatant without disturbing the pellet.
12. Add 100 μL of 75% ethanol/100 μL of TRI reagent to wash the pellet.
13. Centrifuge at 7500 g for 5 min at 4–25°C.
14. Remove all the ethanol carefully without disturbing the pellet.
15. Air dry the pellet for 5 min.
16. Dissolve the RNA in 20 μL of nuclease-free water and mix vigorously.
17. Store on ice for immediate analysis, or place at −80°C for long-term storage.

3.6.3. Assessment of RNA concentration and quality

Assess the RNA yield and quality by spectrophotometry using a nanodrop. RNA concentration can be assessed by measuring its absorbance at 260 nM.

1. Use 1–2 μL of non-diluted sample and read the absorbance at 260 nm(A260) to measure RNA concentration (C). C = A260*40 μg/mL.
2. To assess purity of the extracted RNA, note the A260/A280 ratio given by the nanodrop: it should be between 1.8 and 2.2.

3.6.4. Reverse transcription

To create cDNAs form the extracted mRNAs, use the Origene First Strand cDNA synthesis system for qPCR, following the manufacturer's instructions. Keep your mRNAs and all the components of the kit (except the enzyme) on ice.

1. In thin-walled PCR tubes, prepare a 20 μL reaction mix including 1 μg of extracted mRNA, 4 μL of 5× cDNA synthesis mix, and 1 μl of reverse transcriptase (take it out of the −20°C freezer at the very last minute). Adjust the total volume to 20 μL with the provided nuclease-free distilled water.
2. Mix gently and spin down to collect contents.
3. Place the tubes in a thermocycler programmed as follows: 1 cycle at 22°C for 5 min, 1 cycle at 42°C for 30 min, 1 cycle at 85°C for 5 min, hold at 4°C. At this point, you can store the cDNAs at −80°C.

3.6.5. Real-time quantitative PCR

To perform real-time quantitative PCR, you will need to design a set of primers specific to the transcript of the gene(s) of interest. Primer design is beyond the scope of this review and will not be described here.[12]

1. Prepare a 10 μM primer mix containing an equimolar concentration of forward and reverse primers in ddH_20
2. Dilute the template cDNAs 11-fold by adding 200 μL of PCR-grade water to the 20 μL product of the reverse-transcription.
3. Prepare the qPCR reaction mix in a 96-well plate as follow: Total reaction volume: 25 μl = 11 μL of diluted template cDNA +1.5 μL of 10 μM forward/reverse primers mix +12.5 μL of 2× SYBR green mix. The 2× SYBR green mix is a buffer containing all the components necessary for DNA amplification and detection: Taq DNA polymerase, dNTPs, Mg^{2+} and the DNA intercalating dye SYBR Green. Run each sample in duplicate or triplicate. To detect DNA contamination, always include a negative control in each run. To prepare this control, replace template cDNA with PCR-grade water.
4. Place the 96-well plate in the real-time PCR cycler.

[12]Note that several programs for primer designing are freely available on the web such as primer blast: http://www.ncbi.nlm.nih.gov/tools/primer-blast.

5. Set the detection channel to SYBR Green and the reaction volume at 25 μL.
6. Run the reaction as follow: 1 cycle at 95°C for 10 min to activate the Taq DNA polymerase, followed by 40 cycles of amplification: 95°C for 15 s, 58–60°C (the optimum temperature must be determined for each primer set) for 30–60 s, 72°C for 30–60 s.
7. Repeat the same procedure with a set of primers designed to amplify a control housekeeping gene or other species that should not be affected by your experimental conditions. In studies involving cell death induced by PD toxins (6-OHDA, MPP+), we found that 18S rRNA is a good control and can be used for normalization. At the end of the reaction, proceed to quantification and analysis.

3.6.6. Quantification and analysis

Real-time monitoring of the PCR reaction displays the amount of fluorescence signal emitted from the SYBR green dye. During the amplification reaction, the SYBR green dye is progressively inserted in the newly synthesized double-stranded DNA fragments. The amplified fragments correspond to the region of the cDNA from the gene of interest flanked by the forward/reverse primers binding sites. The initial copy number can be quantitated during real-time PCR analysis based on the threshold cycle (Ct). Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background. The more template is initially present, the fewer number of cycles is needed to reach a threshold at which the fluorescence is statistically significant above background. To calculate the fold induction of your gene of interest (GI) normalized to a control housekeeping gene (HKG) in an experimental condition (Exp) compared to a control condition (Ctl), use the following formula:

$$\text{fold induction} = 2^{-\left[\left(Ct_{GIExp} - Ct_{HGKExp}\right) - \left(Ct_{GICtl} - Ct_{HKGCtl}\right)\right]} \quad (1)$$

3.7. Downstream application: Western blot

3.7.1. Harvesting cells and extracting proteins

1. Take the cells out of the incubator and place them on ice.
2. Remove the medium gently and add 1× cell lysis buffer supplemented with protease inhibitor (30 μL of buffer/cm^2 of culture dish area).
3. Use a cell lifter to detach the cells from the bottom of the well.
4. Pipette the cell lysate into labeled microcentrifuge tubes and place them on ice.
5. Pipette up and down 2–3 more times and briefly vortex to further homogenize the cell lysate. Check the homogenate: if cellular debris is still visible, proceed to sonicate the samples.
6. Sonicate for 20 s (10 pulses over 10 s followed by one continuous 10-s pulse) on ice. At this point, the homogenate should be clear of debris.
7. Store the samples at −80°C before measuring the protein concentration in each sample.

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3.7.2. Assessment of protein concentration and sample preparation

1. Use a technique of your choice to measure protein concentration. We recommend using Thermo Scientific's BCA assay following the manufacturer's instructions (Thermo Scientific #23225).
2. Label a new set of tubes in order to prepare the samples in loading buffer. The following steps (sample preparation, gel electrophoresis and protein transfer) are made using the NuPAGE Novex system from Life Technologies. Please refer to the manufacturer's protocol for further information.
3. In each tube add: Protein sample (to achieve a final concentration of 1–2 μg/μL of protein), 10× dithiothreitol (NuPAGE reducing agent, 500 mM dithiothreitol), 4× loading dye and ddH_2O up to the desired volume.
4. Samples can be stored at −20°C at this point.

3.7.3. Gel electrophoresis

Gel electrophoresis is achieved with the NuPAGE Novex Mini system with Bis-Tris precast polyacrylamide gels.[13]

1. Prepare 1 L of 1× MOPS running buffer (50 mL of 20× MOPS running buffer in 950 mL of ddH_2O).
2. Insert the precast gel in the gel box and fill the inside chamber with 1× running buffer.
3. Boil protein samples for 5 min.
4. Remove bubbles and residues from the gel wells by pipetting.
5. Load 4 μL of molecular weight markers and the protein samples in the desired order.
6. Run the gel under constant voltage at 100–140 V (400 mAmps) for 30–45 min.
7. Monitor frequently to assess the degree of separation and to make sure that the protein samples are not running out of the gel.

3.7.4. Protein transfer

1. Prepare 2 L of 1× transfer buffer (100 mL of 20× transfer buffer with 15% ethanol (300 mL) and 1600 mL of ddH20).
2. Cut a polyvinylidene fluoride (PVDF) membrane (pore size 0.2 μm) and 2 pieces of filter paper to the appropriate dimensions of a transfer cassette, or use a premade sandwich (Biorad).

[13]This system is optimized for separation and resolution of small- to medium-sized proteins (1–200 kDa) under denaturing gel electrophoresis conditions. These precast gels are available in different polyacrylamide percentages, well formats and thicknesses and must be chosen according to specific experimental needs. Acrylamide is a toxic compound: manipulate with caution and wear gloves at all time.

3. Soak the PVDF membrane for few seconds in 95% ethanol then soak in transfer buffer for 5 min.
4. Soak filter paper and sponges in transfer buffer for 5 min.
5. Prepare a "transfer sandwich" with 2–3 sponges, 1–2 pieces of filter paper, polyacrylamide gel containing the separated proteins, PVDF membrane, 1–2 pieces of filter paper, 2–3 sponges. Keep the sandwich wet at all times and avoid the formation of bubbles in between layers.
6. Place the sandwich in a transfer cassette and fill up the gel box with 1× transfer buffer. Apply a constant voltage at 40 V (400 mAmps) for 94 min to transfer the proteins form the gel to the membrane.

3.7.5. Protein detection

1. Check the uniformity and overall efficiency of the transfer by staining the membrane with Ponceau S dye (0.1% w/v Ponceau, 5% acetic acid in ddH_2O).
2. Wash the membrane with TBST (1× TBS + 0.1% Tween) for 5 min. Wash again quickly with TBST to discard the excess of Ponceau.
3. Block non-specific protein binding by incubating the membrane with TBST +5% powdered milk for 45 min.
4. Rinse 3 times for 5 min with TBST.
5. Incubate the membrane with a solution of primary antibody in blocking solution under gentle agitation at 4–25°C from 30 min to overnight.[14]
6. Wash the membrane with TBST 3 times for 5–15 min.
7. Incubate the membrane with a secondary antibody targeted to the primary antibody and bound with horseradish peroxidase.
8. Incubate at room temperature for 1–2 h on a shaker.
9. Wash the membrane with TBST three times for 5–15 min.
10. Incubate the membrane with a chemiluminescent agent.
11. Expose a light-sensitive autoradiographic film against the membrane and develop to reveal the protein signal.
12. Repeat the same procedure with a primary antibody against a control housekeeping protein that should not be affected by your experimental conditions. In studies involving cell

[14]The final concentration of primary antibody as well as the duration and temperature of incubation should be determined empirically and according to the manufacturer's guidelines.

death induced by PD toxins (6-OHDA, MPP+), we found that extracellular signal–regulated kinases (ERK) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are good loading controls and can be used for normalization.

3.8. Downstream application: survival assay

The quantification of viable cells following treatment with PD-toxins and/or gene manipulation can be achieved by several methods. A number of methods (such as MTT or LDH assays) rely on measuring the metabolic activity of the cultured cells and give an indirect and potentially biased measurement of cell viability. We consider that the most accurate and robust measure of cell death is an absolute count of the numbers of surviving cells remaining in the cell culture dish following toxin treatment and/or gene manipulations. We routinely use a method in which the nuclei of the remaining cells are counted by incubating the cell cultures with a detergent solution that lyses the plasma membrane and leaves the nuclei intact.

1. Prepare 100 mL of 10× counting lysis buffer by combining: 5 g of cetyldimethyl-ethanolammonium bromide, 0.165 g of NaCl, 2.8 mL of glacial acetic acid, 50 mL of 10% Triton-X, 2 mL of 1 M $MgCl_2$, 10 mL of 10× PBS, 35.2 mL of H_2O.
2. Take the cells out of the incubator and aspirate all the medium.
3. Add 1× of counting lysis buffer to the wells (250 μL/cm^2 of culture dish area) and leave sit for 5 min.
4. Pipette 10 μL of the suspended nuclei into a hemacytometer and count at least 100 nuclei.
5. Cell survival in a given condition is expressed as the percentage of remaining nuclei in experimental cultures compared to control cultures.

4. Outcomes: examples of findings using the above techniques

4.1. Published findings

Application of the above techniques by our and other groups has identified a variety of transcriptionally regulated genes with potential relevance to the pathophysiology and treatment of PD. Here, we provide a few examples arising from our own studies (**Figure 2**).

An early serial analysis of gene expression (SAGE) study of transcriptional responses of PC12 cells to 6-OHDA treatment revealed a strong endoplasmic reticulum (ER) stress response signature [19, 20]. Such work contributed to the current focus on the role of ER stress in PD [21, 22]. Among the upregulated stress, genes were the transcription factors *ATF4* (Activating transcription factor 4) and *Ddit3* (DNA damage-inducible transcript 3, which encode the ATF4 and CHOP (C/EBP homologous protein) proteins, respectively). *In vitro* work, including with PC12 cells as well as *in vivo* studies have indicated that CHOP is death promoting in the

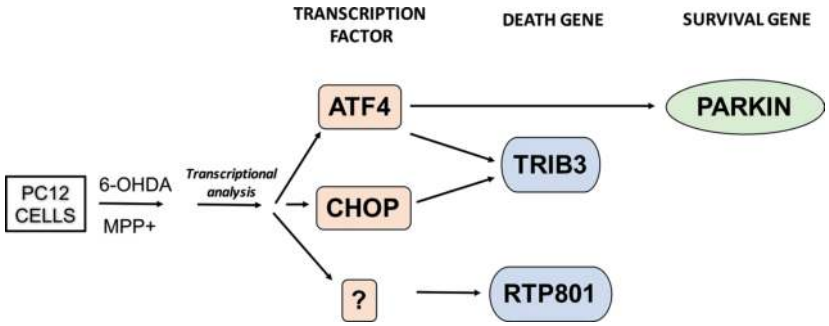

Figure 2. Example of transcriptionally regulated genes in PD cellular models identified by our group using the methods described in this chapter.

context of PD [23–25]. The potential role of ATF4 in contrast has proved to be more complex, and here again, studies with PC12 cells have proved useful for enlightenment. On one hand, ATF4 appears to have protective actions in PD models and does so by causing stabilization of the anti-death protein Parkin [13]. On the other hand, ATF4 appears to cooperate with CHOP in transcriptional induction of Trib3, a protein with pro-apoptotic actions in neurons [25, 26]. *Trib3* was among the genes found to be induced by 6-OHDA [19, 20, 25] in PC12 cells, and subsequently, Trib3 protein was found to be elevated in dopaminergic neurons of PD patients [25]. Down-regulation of Trib3 or inhibition of its transcription is highly protective in multiple cellular PD models including PC12 cells, suggesting it as a potential therapeutic target for PD treatment [25]. An additional transcriptionally regulated gene of interest to arise from the initial SAGE study was *Ddit4* (DNA-damage-inducible transcript 4), which encodes the pro-death RTP801 protein. A series of studies has indicated that like Trib3, RTP801 is elevated in dopaminergic neurons of PD patients and that eliminating its expression is protective in cellular models of PD such as PC12 cells [27–29]. Interference with RTP801 induction in an animal model of PD proved to be protective, thus identifying this as an additional potential target for PD therapy [29].

4.2. Nupr1 is induced but does not regulate Trib3 upregulation in a PD cellular model

As noted above, we recently described Trib3 as a gene-mediating cell death and degeneration in PD and identified two transcription factors, ATF4 and CHOP, responsible for Trib3 upregulation in PD cellular models, including neuronal PC12 cells treated with 6-OHDA [25]. However, ATF4 and CHOP downregulation only partially abrogated Trib3 induction in response to PD toxin mimetics, indicating that other transcription factors might be responsible for Trib3 induction in these models. Several reports indicate that Nupr1 (also known as p8) is upregulated and leads to apoptosis of cancer cells through activation of the ATF4/CHOP-Trib3 pathway [30, 31]. Therefore, we tested whether Nupr1 was also upregulated in PD cellular models and, if so, whether it could regulate Trib3 expression (**Figure 3**).

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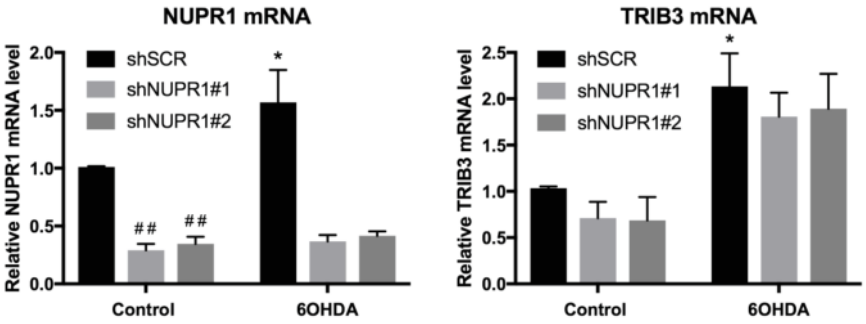

Figure 3. Nupr1 is upregulated but does not regulate Trib3 expression in PD cellular models. PC12 cells were differentiated with NGF for 3 days and infected with three different lentiviruses: two lentiviruses carrying constructs expressing shRNAs directed against two distinct regions of the Nupr1 mRNA (shNUPR1#1 and shNUPR1#2) or a control lentivirus carrying a scrambled shRNA sequence (shSCR). 4 days later, the cells were treated with sterile water (control) or 150 μM 6-OHDA for 8 h (6OHDA). At the end of the treatment, total mRNAs were extracted and RT-qPCR was performed to measure Nupr1 and Trib3 mRNA levels. Nupr1 and Trib3 mRNA levels were normalized to GAPDH. For Nupr1 RT-qPCR primers: (forward) GGGCAAGTTAGGAGCGAGAA and (reverse) GGGCATCCAGTTTTTCCCAC. For Trib3 RT-qPCR primers: (forward) GTTGCGTCGATTTGTCTTCA and (reverse) CGGGAGCTGAGTATCTCTGG. Values are expressed as mean ± SEM of three independent experiments. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test. $^{*}p < 0.05$ comparing shSCR/6OHDA relative to shSCR/control; $^{\#}p < 0.005$ comparing shNUPR1#1/control or shNUPR1#2/control relative to shSCR/control.

Consistent with the upregulation of Nupr1 seen in apoptotic cancer cells [30, 31], we found that 6-OHDA induced a significant 1.6 fold increase in Nupr1. Under these conditions, we measured a 2.1-fold increase in Trib3 mRNA levels, as reported previously [25]. To assess whether Nupr1 is required for this Trib3 induction, we employed lentivirally delivered Nupr1 shRNAs as described in this chapter. Although both shRNA constructs achieved a 70% reduction of Nupr1 mRNA levels, Nupr1 knockdown had no effect on Trib3 mRNA levels neither at baseline nor under 6-OHDA treatment. These results suggest that although Nupr1 is upregulated in PD cellular models, it is not one of the transcription factors responsible for orchestrating Trib3 upregulation in these models.

4.3. xCT/SLC7A11 is induced in PD cellular models

Our PC12 cell experiments indicate upregulation of ATF4 in multiple PD models, and that while ATF4 can play a protective role in PD by reducing loss of the anti-death protein Parkin, it also contributes to induction of the pro-apoptotic protein Trib3 (**Figure 2**). An additional way that ATF4 could affect cell survival or death is by transcriptional regulation of xCT protein (product of the *SLC7A11* gene) [32]. xCT protein levels are reported to be increased in animal PD models such as 6-OHDA and chronic MPTP treatment [33, 34]. These findings are capitulated in our PC12 cell studies, in which we found massive upregulation of xCT mRNA in both the MPTP and 6-OHDA models (**Figure 4**).

xCT is a subunit of the cystine/glutamate antiporter system that transports cystine into cells in exchange for exported glutamate. This property has suggested the possibility of both pro-survival

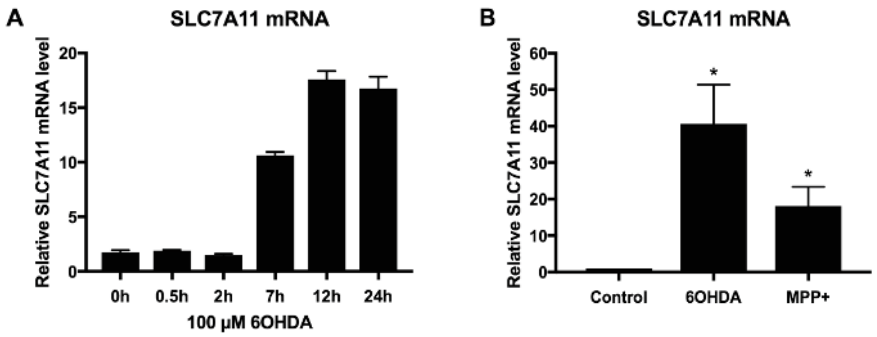

Figure 4. xCT/SLC7A11 mRNA is upregulated in differentiated PC12 cells by PD-mimetic toxin treatment. Neuronally differentiated PC12 cells were treated with 100-μM 6-OHDA for different times as indicated (A) or with 150 μM 6-OHDA for 10 h or 1 mM MPP+ for 16 h (B). Total mRNA was extracted, and RT-qPCR was performed to measure SLC7A11 mRNA levels (normalized to tubulin). For xCT RT-qPCR primers: (forward) GACAGTGTGTGCATCCCCTT and GCATGCATTTCTTGCACAGTTC (reverse). Values are expressed as mean ± SEM. In B, statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. $^{*}p < 0.05$ comparing 6-OHDA or MPP+ relative to control.

and pro-apoptotic activities in the context of PD. By promoting cystine uptake, xCT increases synthesis of glutathione (GSH), an intracellular antioxidant that has been suggested to play a protective role in PD [35, 36]. One possibility is that the upregulation of xCT might be a compensatory protective reaction to increased oxidative stress. Consistent with this idea, treatments that elevate neuronal levels of GSH show protection in a variety of PD models [36, 37]. On the other hand, as an exchanger, xCT elevates extracellular levels of glutamate that in turn may have toxic effects on dopaminergic neurons via glutamate receptors. In agreement with this possibility, 6-OHDA-treated $xCT^{-/-}$ mice were reported to have less striatal extracellular glutamate than wt mice, and their dopaminergic neurons showed substantial protection from 6-OHDA [38]. Such findings raise the challenge of sorting out and appropriately manipulating the pro- and anti-apoptotic actions of xCT for therapeutic advantage in PD. Cellular models of PD with induction of xCT such as described here, have the potential to serve as convenient, first-line screening systems to this end.

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Abbreviations

6-OHDA: 6-hydroxydopamine

ATF4: activating transcription factor 4

BDNF:	brain-derived neurotrophic factor
BSA:	bovine serum albumin
CHOP:	C/EBP homologous protein
ddH_2O:	double distilled or deionized water
DDIT3:	DNA damage-inducible transcript 3
DDIT4:	DNA-damage-inducible transcript 4
dibutyryl cAMP:	dibutyryl cyclic adenosine monophosphate
DMSO:	dimethyl sulfoxide
ER:	endoplasmic reticulum
ERK:	extracellular signal–regulated kinases
FBS:	fetal bovine serum
GAPDH:	glyceraldehyde 3-phosphate dehydrogenase
GFP:	green fluorescent protein
GSH:	glutathione
HBSS:	Hank's balanced salt solution
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+:	1-methyl-4-phenylpyridinium
NGF:	nerve growth factor
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PD:	Parkinson's disease
pen/strep:	penicillin/streptomycin
PVDF:	polyvinylidene fluoride
qPCR:	quantitative polymerase chain reaction
rhBDNF:	recombinant human brain-derived neurotrophic factor
RT-PCR:	reverse transcription polymerase chain reaction
SAGE:	serial analysis of gene expression

TBS:	Tris-buffered saline
TBST:	Tris-buffered saline with Tween
Trib3:	Tribbles pseudokinase 3

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