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Cell-to-Cell Transmission of α-Synuclein Aggregates

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Abstract

It is now recognized that the cell-to-cell transmission of misfolded proteins such as α-synuclein contributes to the neurodegenerative phenotype in neurological disorders such as idiopathic Parkinson's disease, Dementia with Lewy bodies, and Parkinson's disease dementia. Thus, establishing cell-based models for the transmission of α-synuclein is of importance to understand the mechanisms of neurodegeneration in these disorders and to develop new therapies. Here we describe methods to study the neuron-to-neuron propagation of α-synuclein in an in vitro setting that also has in vivo applications.

Keywords

Neurodegeneration; Parkinson's disease; Lewy body disease; α-Synuclein; Propagation; Protein dissemination; Neurons

1. Introduction

Accumulation of protein aggregates across specific brain regions is now recognized as a critical characteristic in major neurodegenerative disorders (1). In Alzheimer's disease (AD), amyloid β (Aβ) protein and tau accumulate in extracellular and intraneuronal compartments (2–4); in Parkinson's disease (PD), the synaptic protein α-synuclein accumulates in axons and neuronal cell bodies (5, 6); in Huntington's disease (HD) and other expansion diseases, polyglutamine (polyQ) proteins accumulate in the nucleus and the cytoplasm (7); and misfolded prions accumulate in the neuropil in Creutzfeldt-Jakob disease (CJD) (10). Neurodegeneration in most of these disorders probably initiates at the synaptic site where discrete protein aggregates denominated oligomers impair neuronal transmission and functioning (8–10). While oligomers are usually diffusible, non-fibrillar, small order aggregates, larger polymers in the form of amyloid fibrils comprise the inclusion bodies characteristic of these disorders and are now believed a pathway for sequestration of more toxic oligomers (11). Alterations in the balance of protein aggregation, clearance, and synthesis play an important role in the formation of toxic oligomers and in the neurodegenerative cascade (12–17). In addition, recent evidence suggests that protein propagation from cell to cell might play an important role in the mechanisms of neurodegeneration (18–24).

α-Synuclein is a cytoplasmic protein, most abundant at the synaptic terminals (25–28), is a 140 kDa molecule with an N-terminus that binds lipids, a central hydrophobic region (NAC) with amyloidogenic capacity and an acidic C-terminus tail (29). Mutations, multiplications, and polymorphisms in α-synuclein have been linked to familial and sporadic forms of Parkinson's disease and Lewy body disease (30). Lewy body disease is an heterogenous

group of disorders that includes idiopathic Parkinson's disease, Dementia with Lewy bodies, and Parkinson's disease dementia (31, 32).

In addition to the oligomeric toxic α-synuclein that is retained in the neurons and cell membranes, a small amount of α-synuclein is released from cells, apparently in the absence of serious membrane damage (33). The release of α-synuclein oligomers from neurons is increased under disease conditions and leads to worsening of the synaptic damage and cell death via apoptosis (22, 24). This cell-to-cell transmission of α-synuclein may account for the recent reports showing the spread of α-synuclein with the formation of Lewy bodies from host tissues to long-term fetal cell grafts in Parkinson's patients (21).

For example studies utilizing both oligomeric and fibrillar forms of α-synuclein are taken up by neuronal cells through endocytosis, thereafter targeted to the lysosome for degradation (22, 23). We have demonstrated the dissemination of α-synuclein aggregates through cell-to-cell transmission (20). This study showed that α-synuclein aggregates can be transferred from aggregate-producing cells to aggregate-free cells via endocytosis of the recipient cells. This transmission became more efficient when the lysosomal function was compromised in the recipient cells, suggesting the antagonizing role of lysosomes against the aggregate transmission. When mouse neuronal progenitor cells were transplanted into the brains of transgenic mice overexpressing human α-synuclein, α-synuclein proteins were transferred from host neurons to the grafted cells. Moreover, we have also shown that α-synuclein aggregates can be transferred from neurons to astroglial cells leading to a proinflammatory reaction with toxic consequences (34).

Understanding the mechanisms of cell-to-cell propagation of α-synuclein aggregates is under investigation. While some studies propose a role for exocytosis and endocytosis, others have suggested that α-synuclein might disseminate via a transynaptic pathway, extrusion after cell death, or nanotube formation (24). Given the important role in disease spreading, neurodegeneration, disease progression, and graft dysfunction understanding the cellular mechanisms of α-synuclein aggregates dissemination in in vitro models is of significant importance. Moreover, dissemination in in vitro models are useful for therapeutical development. In this context, this chapter provides some key protocols to study the process of α-synuclein dissemination in neuronal cultures.

2. Materials

This chapter provides protocols to investigate: (1) the uptake and propagation of tagged recombinant α-synuclein in neuronal stem cells; (2) production and infection of neuronal cells with lentiviral vectors expressing myc-tagged α-synuclein and (3) studies of neuron-to-neuron dissemination of myc-tagged α-synuclein.

2.1. Recombinant α-Synuclein in Neuronal Stem Cells

2.1.1. Cell Culture

1. Cryopreserved mouse cortical neural stem cells (Millipore).

2. DMEM/F12 w/o HEPES, w/L-Glutamine (Mediatech);L-Glutamine (1,000×); PenStrept (1,000×), both from Gibco.
3. B27 supplement (w/o vitamin A, 500×); FGF2; EGF and Heparin (all from Invitrogen).
4. Acid-treated glass coverslips, 12-well cell culture plates, poly-L-ornithine (10 mg/mL), laminin (1 mg/mL) both from Sigma.
5. Paraformaldehyde (4% in PBS).

2.1.2. α-Synuclein Labeling

1. Human recombinant α-synuclein (Calbiochem).
2. Zeba™ Desalt Spin columns (Pierce).
3. Alexa Fluor® 555 Microscale Protein Labeling kit (Invitrogen).

2.2. α-Synuclein myc Lentiviral Vector Productionfor Infection of Neurons and Propagation Studies

The “packaging” plasmids (Gag-Pol, VSV-G, Rev) should be purified by an endotoxin-free plasmid-prep kit. The DNA concentration should be determined by $A^{260/280}$ and confirmed by gel-electrophoresis. Verify the DNA preparations by restriction digestion analysis, and aliquot the DNA at 1 μg/μl in endotoxin-free TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.2.1. Plasmid Preparation

1. Poly-L-lysine, store 4°C. Dilute to 0.01% in 1× DPBS.
2. Reagents for 2× BBS, filter sterilize (0.22 μm), store room temperature.

500 mM BES	10.66 g/100 ml
2.8 M NaCl	16.35 g/100 ml
150 mM Na_2 PO_4	2.1 g/100 ml

2.2.2. Reagents/Buffers

3. 500 ml 2× BBS, store 4°C.

50 ml 2.8 M NaCl

50 ml 500 mM BES

5 ml 150 mM Na_2HPO_4

Add water (UltraPure, Invitrogen) to 450 ml

(a) Adjust pH to 6.95 with 1 M NaOH

(b) Add water to 500 ml

(c) Filter sterilize (0.22 μm)

(d) Store at 4°C for 3–4 days prior to validation.

4. 100 ml 10× $CaCl_2$, store −20°C (will not freeze)

2.5 M $CaCl_2$, 36.75 g/100 ml

Dissolve and filter sterilize (0.22 μm) using syringe.

Store in 1.4 ml aliquots. Do not freeze/thaw.

5. 20% sucrose, store −20°C

Dissolve 20 g sucrose in 100 ml HBSS.

Filter sterilize (0.22 μm) using syringe.

Store in 10 ml aliquots.

2.2.3. Validation of 2× BBS/$CaCl_2$: Combination—Transfect a 10-cm dish of 293T cell (70–80% confluent) with 20 μg GFP reporter plasmid.

Observe transfection efficiency 48 h later (should be at least 90–95% GFP + cells).

Aliquot 2× BBS 14 ml per tube and store at 4°C.

2.3. Studies of Neuron-to-Neuron Dissemination of myc-Tagged α-Synuclein

1. Retinoic acid (100 mM in DMSO).
2. SH-SY5Y cells.
3. Myc-tagged α-synuclein lentiviral vectors.

3. Methods

3.1. Recombinant α-Synuclein in Neuronal Stem Cells

3.1.1. Labeling of a-Synuclein

1. To ensure proper labeling of the protein, replace the buffer from α-synuclein stock solution for PBS using desalting columns (Zeba™ Desalt Spin columns). Following the manufacturer's instructions, wash columns four times with 100 μL PBS. After washing, load 100 μL of α-synuclein stock in the column and centrifuge at 1,500 × *g* for 1 min. Protein is now in PBS, ready for labeling and should be at a concentration of 1 mg/mL.
2. For labeling of proteins with Alexa 555, follow the instructions of the manufacturer. To calculate the amount of nanomoles of dye that should be added per nanomole of α-synuclein, consider that optimal degree of labeling for α-synuclein is 3 (according with manufacturer's indications, e.g., for labeling of 100 μg of a protein with an MW of 14 kDa, you will need 2.64 μL of stock dye per sample). Transfer up to 100 μL of α-synuclein (1 mg/mL) to a reaction tube. Add 10 μL of 1 M sodium bicarbonate and mix. Add 2.64 μL of reactive dye solution and mix thoroughly by pipetting up and down. Incubate at room temperature for 15 min. Pack the resin in the supplied columns as indicated in the

instructions. Load 50 μL of conjugate reaction mix per column and centrifuge at 16,000 × *g* for 1 min. Determine protein concentration and degree of labeling by reading absorbance at 280 and 555 nm and applying the following equations:

$$\text{Protein concentration (mg/mL)} = \frac{[\text{A}^{280} - 0.08\,(\text{A}^{555})] \times \text{dilution water}}{\text{A}^{280} \text{ of protein at 1 mg/ml}},$$

where the divisor could be replaced by the molar extinction coefficient of the protein at 280 nm, which for α-synuclein = 0.354.

$$\text{Degree of labeling (moles dye/mole protein)} = \frac{\text{A}^{555} \times \text{dilution water}}{150,000 \times \text{protein concentration (M)}}.$$

Labeled α-synuclein should be stored protected from light. For short-term use, store it at 4°C. Alternatively the stock can be aliquoted and stored at −20°C for a few months. Avoid repeated freezing and thawing.

3.1.2. In Vivo Testing of α-Synuclein Uptake

1. Prepare Basal culture media by supplementing DMEM/F12 with L-glutamine, B27 and PenStrept. Filter the media. Freshly prepare aliquots of Expansion media by adding FGF2 (20 ng/ mL), EGF (20 ng/mL), and heparin (20 μg/mL) to 50 mL of Basal media. Expansion media can be used for 2 weeks after preparation.
2. Coat culture plates and coverslips with poly-L-ornithine and laminin. Prepare a 1:200 solution of poly-L-ornithine in H_2O and cover the surface of the plates (use 1 mL/well for a 12-well plate containing glass coverslips. Push the covers to the bottom with a pipette tip to ensure that they are well submerged in the solution). Incubate overnight at room temperate in the tissue culture hood. The following day discard the poly-L-ornithine solution and wash the plates two times with PBS before coating with Laminin (1:200 dilution in PBS). Incubate overnight at room temperate in the tissue culture hood. When coating is done, seal plates and flasks tightly with tape and store at −20°C for up to 6 months. Before use, thaw the laminin, aspirate, and wash once with PBS before plating cells.
3. Grow mouse cortical neural stem cells (MCNSCs) in expansion media, in a 37°C incubator with 5% CO_2. Change media every other day, splitting the cells when they reached 80% confluency. Plate 3×10^4 cell/mL in each cover and incubate overnight.
4. Freshly dilute the necessary amount of Alexa 555-labeled α-synuclein (from Subheading 2.1) in expansion media to obtain a final concentration of 0.3 μM α-synuclein. Incubat the cells in α-synuclein-supplemented media for 24 or 48 h. Finally fix the cells with 4% paraformaldehyde in

PBS and keep the fixed covers protected from light at 4°C until processing for microscopy.

3.2. α-Synuclein myc Lentiviral Vector Productionfor Infection of Neurons and Propagation Studies

3.2.1. Virus Production Planning—Day 1: Coat plates with poly-L-lysine for 15 min at room temperature, aspirate remaining solution.

Plate HEK293T cells so that 24 h later cells will be 70–75% confluent.

Day 2: Transfect cells with packaging plasmid and vector plasmid (according to protocol below).

Day 3: Change medium.

Day 4: 1st collection, refeed cells. Centrifuge supernatant (according to protocol below).

Day 5: 2nd collection.

Centrifuge supernatant.

Purify virus (according to protocol below).

3.2.2. Transfection/ Collection Procedure

	10 cm plate	15 cm plate	12 × 15 cm plates	24 × 15 cm plates
"Vector"	10.0 μg	22.5 μg	270 μg	540 μg
pGag/Pol	6.5 μg	14.6 μg	176 μg	352 μg
pRev	2.5 μg	5.6 μg	68 μg	136 μg
pVSV-G	3.5 μg	7.9 μg	95 μg	190 μg
H_2O	QS 450 μl	QS 900 μl	QS 12.15 ml	QS 24.3 ml
$CaCl_2$ (10×)	50 μl	100 μl	1.35 ml	2.7 ml
2× BBS	500 μl	1 ml	13.5 ml	27 ml

1. Mix the 4 plasmids according the scheme above.
2. Add H_2O.
3. Add $CaCl_2$, mix thoroughly.
4. Add 2× BBS, mix gently by inverting ~3–5 times, and incubate for 15 min at RT.
5. Add the DNA mix drop wise to dish (2.2 ml per 15-cm plate, 1.0 ml per 10-cm plate), try to spread DNA by carefully rocking the plate.
6. Place the cells in a humidified 37°C incubator at 3% CO_2.
7. Change medium next morning and incubate at 37°C at 10% CO_2.

8. One and 2 days later collect and filter the media through a 0.22-μm cellulose acetate vacuum filter.

3.2.3. Centrifugation

1. Load filtered supernatant into centrifuge tube.
2. Mark tube on one side to indicate location of pellet after centrifugation. This is especially important when using Optimem as the virus pellet is nearly impossible to see.
3. Centrifuge 2 h, 50,000 × *g* at room temperature. If using a fixed angle router, place tubes so mark is facing outward so that the virus pellet can be easily located following centrifugation.
4. Mark location of virus pellet on tube prior to removing supernatant.
5. Discard supernatant and invert tubes on a paper towel. Aspirate excess medium.
6. Resuspend pellets from all tubes in 1,000 μl HBSS. Repeat for collection from day 2 and pool supernatant. Virus from day 1 can be stored at 4°C overnight.
7. Load pooled particles (now in a volume of 2 ml) on 2 ml 20% sucrose in centrifuge tubes. Load very slowly to avoid disrupting sucrose cushion. Wash remaining supernatant from tube with additional 500 μl HBSS and add to centrifuge tube.
8. Centrifuge 2 h, 50,000 × *g* at room temperature.
9. Discard supernatant, resuspend pellet in 250–500 μl HBSS and transfer viral particles into microcentrifuge tube with screw cap and rubber O-ring.
10. Wrap tube with parafilm and shake on vortex at low speed for 1 h at room temperature to resuspend virus.
11. Quick-spin in microcentrifuge (~15 s) and aliquot supernatant in 10–50 μl aliquots into microcentrifuge tubes containing screw cap and rubber O-ring and store at −80°C.

3.2.4. p24-Titer Determination by Perkin-Elmer p24-ELISA—This protocol is basically carried out according to the instructions provided by Perkin-Elmer.

Standards:

Tube	Standard well	p24 (pg/ml)	Preparation
A		4,000	245 μl HBSS + 5 μl control p24 from kit
B	C	100	780 μl HBSS + 20 μl

Tube	Standard well	p24 (pg/ml)	Preparation
C	D	50	200 µl HBSS + 200 µl
D	E	25	200 µl HBSS + 200 µl
E	F	12.5	200 µl HBSS + 200 µl
F	G	6.3	200 µl HBSS + 200 µl
G	H	3.1	200 µl HBSS + 200 µl

Sample dilutions

Tube	Dilution	Preparation
1	10^{-3}	1,500 µl HBSS + 1.5 µl virus sample
2	10^{-5}	980 µl HBSS + 10 µl 10^{-3} + 10 µl Triton
3	10^{-6}	900 µl HBSS + 100 µl 10^{-5}
4	10^{-7}	900 µl HBSS + 100 µl 10^{-6}
5	10^{-8}	900 µl HBSS + 100 µl 10^{-7}

ELISA

1. Prewash strips 3× with wash buffer from kit.
2. Load 200 µl samples/standard per well and incubate at 37°C for 2 h (or 4°C overnight).

   (a) Load standards in column 1 as below.

   (b) Load samples in next column in wells A–D or E–H from 10^{-5} to 10^{-8}.

   - A—Empty
   - B—200 µl HBSS
   - C—H as explained above.
3. Dump samples and wash wells 6× with wash buffer.
4. Add 100 µl detector-antibody and incubate at 37°C for 1 h.
5. Dump antibody and wash well 6× with wash buffer.
6. Add 100 µl streptavidin conjugate (10 µl streptavidin per ml diluent) and incubate at 37°C for 30 min.
7. Dump the streptavidin and wash well 6× with wash buffer.
8. Add substrate (1 tablet OPD per 11 ml substrate buffer) and incubate at room temperature for 30 min in the dark.
9. Stop color reaction by adding 100 µl stop solution to each well.

10. Read the plate on a plate reader at 490 nm with a reference filter at 600 nm.

11. Sample concentrations of p24 can be determined by comparing to the standard curve.

3.2.5. Biological Titer Determination by FACS

1. Plate 5×10^4 HEK 293T cells per well of a 24-well plate (0.5 ml each well).

2. 4 h Later, aspirate media from all wells except control samples.

3. Add 200 μl media to each remaining well of cells.

4. Dilute virus as described for the p24 ELISA assay so that you have samples ranging from 10^{-3} to 10^{-8}.

5. To each well, add 100 μl of virus dilution sample from 10^{-3} to 10^{-8} dilution.

6. Incubate for 24 h in a humidified incubator at 37°C with 10% CO_2.

7. Replace media after 24 h and continue incubation for 48 h.

8. Aspirate media, wash with PBS and resuspend cells in 500 μl of PBS for FACS analysis.

9. Calculate the titer a:

   (% Positive cells/100) × (No. of cells) × (dilution factor) × (1, 000 for ml) = (titer/ml),

   i.e.,(0.0223transducedcells)×(50,000cells) × (1,000) × 1,000 μl/ ml = 1.11×10^9 TU/ml.

This can be used to determine the constant of ng p24/TU of virus typical for your virus preparations. Thus future preparations can be tittered solely by the p24 ELISA using the conversion factor identified from the biological titer determined from the production of LV-GFP.

3.2.6. Lentivirus Infection of Cells

1. To calculate the amount of virus required for infection, determine the optimal multiplicity of infection (MOI) for your specific cell line. In other words, how many virus particles are required per cell for optimal infection?

2. The efficiency of virus infection in each cell line must be determined prior to a full experiment as it can vary dramatically. For example, HEK293T cells can be fully infected with an MOI of 10, while some neuronal cells may require an MOI of 50 or more.

3. Once the optimal MOI has been determined, to calculate the volume of virus required use the following equation:

$$\frac{(\text{No. of cells}) \times (\text{MOI})}{\text{Concentration of virus (titer)}} = \text{volume of virus required,}$$

where the concentration of the virus is expressed as transducing units (tdu)/ml (or infectious virus/ml). For example, I have a plate of 1×10^8 cells that I want to infect at an MOI of 50 and my virus has a titer of 5×10^9 tdu/ml.

$$\frac{(2.5 \times 10^6 \text{ cells}) \times 50 \text{ } MOI}{5 \times 10^9 \text{ tdu/ml}} = 0.025\text{ml} \quad (25\mu\text{l of virus})$$

Conventionally, cell are counted and plated a few hours before infection just to allow the cells to settle and attach to the dish. Then, the virus is added in a volume of media equal to 1/10 of the total volume of media in the dish. Gene expression usually begins 24–48 h after infection but this depends on the cell type used. It is recommended to avoid freeze/thaw any virus aliquot more than twice since virus infection will be dramatically reduced.

4. Studies of Neuron-to-Neuron Dissemination of myc-Tagged α-Synuclein

1. Setting of SH-SY5Y cells.
   - – Acceptor cells: Split SH-SY5Y cells to coverslips in a 12-well plate (1×10^4).
   - – Donor cells: Split SH-SY5Y cells (1.5×10^5 /dish) to a 35-mm dish.
2. Differentiation (day 0).
   - – Change medium with 50 mM all L-*trans* Retinoic acid the next day.
   - – Change medium with retinoic acid (50 mM) every 2–3 days.
3. Infection of donor cells with recombinant lentiviral vectors (day 5).
   - – Add lentiviral vector (m.o.i. 30) in 1/2 volume of culture medium.
   - – 37°C, 1.5 h.
   - – Add 1/2 volume of culture medium with retinoic acid.
   - – Rinse cells three times with warm DMEM.
4. Labeling of acceptor cells with Qtracker595 (Invitrogen) (day 6).

– Mix component A and B (1:1).

– RT, 5 min.

– Dilute mixture with fresh medium (1:1,000).

– Vortex vigorously for 30 s.

– Replace medium of acceptor cells with the Qtracker mixture.

– 37°C, 2 h.

– Wash five times with warm DMEM.

5. Addition of donor cells to acceptor cells (day 6).

– Trypsinize donor cells.

– Resuspend cells in culture medium.

– Remove medium from acceptor cells and add medium containing donor cells (1:12 diluted from a 35-mm dish).

– Culture as usual for 1–3 days.

6. Fixation.

– Wash coverslips twice with cold PBS.

– Fix cells in 4% paraformaldehyde in PBS for 30 min, RT.

– Rinse three times with PBS.

7. Immunofluorescence cell staining.

– Permeabilize cells with ice-cold 0.1% TX-100 in PBS for 5 min RT.

– Rinse again with PBS for three times

– Blocking in PBS/5% BSA/3% Goat serum for 30 min RT.

– Replace with appropriate primary antibodies in blocking solution.

– RT, 30 min.

– Wash three times with PBS—20 min each.

– Add fluorescent dye-conjugated goat anti-mouse antibody in blocking solution.

– RT, 30 min.

– Wash three times with PBS—30 min each.

– TOPRO-3 (Invitrogen) staining of nuclei diluted 1:1,000 in PBS, RT 10 min.

– Wash three times with PBS—5 min each.

– Put a drop of anti-fade reagent (Invitrogen) on slide and cover with coverslip. Wait until hardens (in dark). After drying for several hours, seal with clear nail polish.

– Store at 4°C.

– Images were obtained with an Olympus FV1000 confocal laser scanning microscope, and the fluorescence intensity was quantified using FV10-ASE 1.7 software (Olympus).

5. Note

The experiments involving the transfer of the myc-tagged α-synuclein from the donor to the acceptor cells can be performed as described here plating the cells together or coculturing using chambers or separate coverslips placed in the same dish. The myc-tagged α-synuclein in the acceptor cells can be detected with antibodies against myc or α-synuclein. The tagging of the acceptor cells can be done with dyes as described in the protocol above or with lentiviral-GFP infection. The protocol described here involves transfer from neuron to neuron; however, the same approach can be used for neurons to glial cells and vice versa.

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