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APPENDICES

APPENDIX A

CELL CULTURE

Table 4 Make culture media for BV2 cell lines

Reagent	Volume
DMEM	xxx ml
Antibiotics (penicillin/streptomycin)	10 ml
L-glutamine	20 ml
HEPES	10 ml
Sodium pyruvate (solubilize in 15 ml tube of warm DMEM)	100 mg
Total Volume	1L

Add FBS to get 5% FBS DMEM before use

Table 5 Make culture media for BV2 cell lines (powder media, pH 7.2)

Reagent	Volume
DMEM	1 bag
Sodium bicarbonate	3.7g/L
Adjust pH 7.2	
HEPES	10 ml
Antibiotics (penicillin/streptomycin)	10 ml
Total Volume	1L

Add FBS to get 5% FBS DMEM before use

Splitting Cells (BV2)

1. Aspirate culture media
2. Wash cells with Hanks' solution (10ml/20ml); remove remaining media
3. Aspirate Hanks' solution
4. Add 1x-Trypsin-EDTA (1.5 ml) and incubate at 37°C for 3 min
5. Observe cells, make sure they are floating; tap side of flask to detach cells
6. Neutralize with culture media
7. Transfer to 15 ml conical tube
8. Centrifuge at 200 g for 2 min 30 sec
9. Aspirate supernatant
10. Add 10 ml culture media
11. Split cells (1:5-1:20) with fresh culture media
12. Incubate the cell cultures at 37°C

Freezing Cells (BV2)

Make freezing culture media: 20% FBS, 7% DMSO and DMEM.

Plate vials in Cryo freezing container and store in -80°C overnight, transfer to liquid nitrogen for long storage.

Table 6 Make culture media for HFE-transfected SH-SY5Y cell lines

Reagent	Volume
DMEM/F12	xxx ml
FBS	100 ml
Antibiotics (penicillin/streptomycin/glutamine)	10 ml
MEM Non-essential Amino Acid	10 ml
Sodium bicarbonate (solublilize in 15 ml tube of warm DMEM/F12)	0.3 g
Total Volume	1L

Splitting Cells (SH-SY5Y)

5. Aspirate culture media
6. Wash cells with Hanks' solution (10ml/20ml); remove remaining media
7. Aspirate Hanks' solution
8. Add 1x-Trypsin-EDTA (1.5 ml/3ml) and incubate at 37°C for 3 min
- Stock 10x Trypsin-EDTA; 4ml of 10x Trypsin-EDTA+36 ml of Hanks' solution=40 ml
5. Observe cells, make sure they are floating; tap side of flask to detach cells
6. Neutralize with culture media
7. Transfer to 15 ml conical tube
8. Centrifuge at 2,400 rpm for 2 min 30 sec
9. Aspirate supernatant
10. Add 10 ml DMEM/F12, vortex the tube
11. Centrifuge 2,400 rpm for 2 min 30 sec
12. Aspirate supernatant
13. Split cells (1:5-1:20) with fresh DMEM/F12
14. Add Geneticine (50 μ l/10ml DMEM/F12) for vector and all cell cultures
- Stock Geneticine 50 mg/ml; final concentration 250 μ g/ml
15. Incubate the cell cultures at 37°C

Freezing Cells (SH-SY5Y)

Make freezing culture media: DMEM/F12 media and 20% DMSO

(generate 2 vials/T-150 flask; 1.5 ml/ vial)

Plate vials in Cryo freezing container and store in -80°C overnight, transfer to liquid nitrogen for long storage

APPENDIX B

RT-PCR AND SPECIFIC BUFFER

1. Preparation of the reaction mix for cDNA synthesis

Before starting the experiment thaw all reagents except of the polymerase, mix them thoroughly and centrifuge briefly. Use a sterile microcentrifuge tube to add the reagents to the reaction mix in a fixed order as outlined in the table

Table 7 Preparation of the reaction mix for cDNA synthesis

RT Reacion	Volume/1 sample (μ l)	Volume/2 sample (μ l)	Volume/3 sample (μ l)	Volume/4 sample (μ l)
10X Reaction Buffer	2	4	6	8
25mM MgCl ₂	4	8	12	16
dNTP	2	4	6	8
Random Primer	2	4	6	8
RNase Inhiitor	1	2	3	4
AMV Reverstrascriptase	1	2	3	4
Master Mix	12	24	36	48
Master Mix Aliquot	12	2x12	3x12	4x12
2 ug of RNA (Xi)	Xi	2xXi	3xXi	4xXi
RNase Free Water (20-12-Xi)	(20-12-Xi)	(20-12-Xi)	(20-12-Xi)	(20-12-Xi)
Total Volume	20	2x20	3x20	4x20

i = The number labeled in each sample

2. Preparation of the reaction mix for PCR

Before starting the experiment thaw all reagents except of the polymerase, mix them thoroughly and centrifuge briefly. Use a sterile microcentrifuge tube to add the reagents to the reaction mix in a fixed order as outlined in the table

Table 8 Preparation of the reaction mix for PCR

PCR Reacion	Volume/1 sample (μ l)	Volume/2 sample (μ l)	Volume/3 sample (μ l)	Volume/4 sample (μ l)
10X Reaction Buffer	2.5	5	7.5	10
25mM MgCl ₂	1.5	3	4.5	6
dNTP	0.5	1	1.5	2
Taq	0.2	0.4	0.6	0.8
Forward Primer	0.7	1.4	2.1	2.8
Reward Primer	0.7	1.4	2.1	2.8
Master Mixt	6.1	12.2	18.3	24.4
Master Mix Aliquot	6.1	2x6.1	3x6.1	4x6.1
cDNA	5	2x5	3x5	4x5
RNase Free Water (25-6.1-5)	13.9	2x13.9	3x13.9	4x13.9
Total Volume/sample	25	2x25	3x25	4x25

3. Buffers prepations for RT-PCR

10x Ficoll loading buffer 10 ml

Ficoll	25	g
Bromphenol blue	0.025	g
0.5 M EDTA (pH 8.0)	0.2	ml

Adjust volume to 10 ml with dH₂O, store at -20°C

Tris-EDTA buffer (TE buffer) 10 ml

RNase free water	10	ml
1 M Tris	100	μ l
0.5 EDTA	20	μ l

1.5% Agarose gel (w/v) 100 ml

Agarose	1.5	g
1x TBE	100	ml

Dissolve by heating and occasional mixing until no granules of agarose are visible. Add ethidium bromide (stock 10 mg/ml) 10 μ l (final concentration 1 μ g/ml)

10x TBE buffer (pH 8.0) 1 liter

Tris base	108	g
EDTA 2H ₂ O (pH 8.0)	40	ml
dH ₂ O	800	ml
Slowly add the boric acid, anhydrous	55	g

Adjust the pH to 8.0 with conc.HCl

Adjust the volume to 1 liter with dH₂O

1x TBE buffer 1 liter

10x TBE buffer	100	ml
dH ₂ O	900	ml

Adjust the pH to 7.4 with conc. HCl

Adjust the volume to 1 liter with dH₂O

APPENDIX C

WESTERN BLOT AND SPECIFIC BUFFER

1. Protein assay (Lowry Method)

This assay is designed to quantify 0.125 to 2 mg/ml protein. A standard curve is an absorbance (A) at 750 nm versus BSA concentration (mg/ml) confirm to Beer's Law (it's a straight line passing through the origin). Determine the line of best fit for the data by linear regression ($y = ax+c$) along with an r^2 value. Then use the linear regression equation to determine the protein content of samples based upon absorbance as follows :

Set up a series of standards which contain : 0.125, 0.25, 0.5, 1, 2 mg/ml of BSA. The BSA was prepared as stock at concentration of 2 mg/ml.

1.1 Procedure

5 μ l of five standards dilution, samples and blank tube (lysis buffer) were added into 1.5 ml tubes. Then 25 μ l of solution A were added and mixed by vortexing. After that, quickly added 200 μ l of solution B to each tube, vortexed immediately, and allowed to stand 15 min at room temperature. The absorbance of each solution was read and recorded at 750 nm against a reagent blank by using 1 cm³ cuvettes. The excel spreadsheet and the linear regression equation of a standard curve were used for the Lowry assay to calculate the concentration of protein in each sample.

2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.1 Assembly of apparatus

The reagents used in preparing the gel should be removed from the refrigerator and allowed to warm and degas for 1 h prior to the preparation of the gel. While this is happening, set up the sandwich plates for casting the gel itself. A sandwich consists of

one outer is a rectangular back plate with the rounded bottom corners, measuring 10 x 11.5 glass plate and one inner is same size but with a notch 1.1 cm deep and 10.4 cm long separated by plastic spacers of equal thickness (0.75 mm). In order to prepare a flawless gel, one containing has no air bubble or debris, the glass plates must be perfectly cleaned with liquid detergent, rinse with distilled water and then dry with absolute ethanol. The plates were wiped with gauze. To protect the bubble airs, the edge side of outer glass plates were sealed by gasket. After that, the casting clamps were used to mount the outer and inner glass plates facing together.

2.2 Preparation of Slab gel

The separating gel contain 10% acrylamide was carefully poured between the plates so that it flows down the slide between plates and fill from bottom to top with no air bubbles. The height of the separating gel was adjusted by the comb. Those height of the gel should be approximately 1 cm below the bottom of the comb. The top layer was filled with a small amount of dH₂O using a syringe for aiding the formation of smooth interface. Add water to height of 2-3 mm. The polymerize was allowed to incubate at room temperature for at least 40 min. After the separating gel is solid (polymerize) then the 5% stacking acrylamide gel would be prepared. The water was drained off and excess liquid was removed with a piece of whatman 3 MM paper. The correct comb (number of wells, thickness same as spacers) with ten teeth was immediately gently inserted between the surfaces of lower gel to create lanes for adding sample. It should be made sure that no air bubbles form around the teeth of the comb, as they will impede the migration and separation of the proteins. Then leaving the gel for one hour at room temperature prior to electrophoresis were performed.

2.3 Preparation of sample

While the stacking gel is polymerizing, prepared the sample. Equal amounts of total protein from each sample were mixed with sample buffer. Heating the samples

for 10 min to 100°C in a boiling water bath were performed to denature the protein. The treated protein solution could be kept at 4°C until load on the gel. Unused sample can be stored at -20°C and boiled again before using. The markers do not need to be boiled.

2.4 Electrophoresis

After polymerization is complete, gently remove the comb, being careful not to rip any of the lanes and remove the bottom spacer, mount the gel in the electrophoresis apparatus so that the notch in the inner glass plate was next to and line up with the notch on the upper buffer chamber which had been filled with Tris-glycine electrophoresis buffer, pH 8.3. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. Air bubbles will interfere by causing disruptions in the electrical circuit and an uneven electrophoresis. This is done with a bent hypodermic needle attached to a syringe. Samples were loaded into the bottom lanes by using pipette tips. When loading the wells, be sure that the tip of the gel loading is between the plates and directly over the desired well before dispensing the sample to prevent contamination of neighboring wells. Add 3 μ l of the molecular weight marker. The electrophoresis apparatus was attached to an electric power supply. The power supply was turned on at 125 volt. Small bubbles should start to be produced and rise off the electrode wire at the bottom of the lower chamber. If no bubbles appear then there is an electrical circuit problem. The gel was run until the dye front reaches the bottom of gel, approximately 120 min. Then the power supply was turned off. The orientation of the gel from the glass plate was removed into transfer buffer for transfer protein membrane.

Table 9 Preparation of the solutions for Tris/Glycine SDS-Polyacrylamide Gel Electrophoresis for western blot

Reagents	Separating gel (ml)					4%Stacking gel (ml)
	6%	8%	10%	12%	15%	
H ₂ O	5.3	4.6	4 ml	3.3	2.3	2.1
30% Acrylamide mix	2	2.7	3.3 ml	4	5	0.5
1.5 M Tris (pH 8.8)	2.5	2.5	2.5 ml	2.5	2.5	-
1.0 M Tris (pH 6.8)						0.38
10% SDS	0.1	0.1	0.1 ml	0.1	0.1	0.03
10% APS	0.1	0.1	0.1 ml	0.1	0.1	0.03
TEMED	8 μ l	6 μ l	4 μ l	4 μ l	4 μ l	0.004
Total volume (ml)	10	10	10	10	10	3

3. Protein transfer

Cut one sheet of PVDF membrane and two sheets of absorbent filter paper to the size of the gel. Then, soak the PVDF membrane in methanol for 10 seconds. Immerse membrane, filter papers, and support pads in transfer buffer to ensure they are thoroughly soaked. Be careful to exclude air bubbles from the support pads. Assemble the transfer cassette as follows (black side down): fiber pad, filter paper, gel (no bubbles), PVDF membrane (no bubble), filter paper and fiber pad. Place the complete sandwich in the transfer tank with the membrane closest to the positive electrode (anode, red electrode). The sandwich was transferred for 120 min at 100 volt. After transfer, the membrane is removed from the sandwich and marked the membrane by clipping one corner, following placed into blocking solution.

4. Blocking

After, the protein was transferred to the PVDF membrane, and then it can be used for probing. The sensitivity of western blotting depends on reducing this

background of non-specific binding by blocking potential binding site with irrelevant protein. Place the PVDF membrane in a container, and add blocking solution, incubate for 1 h at room temperature with gentle agitation on a platform shaker. After blocking, the membrane was washed 2 time 10 min, in TBS washing buffer.

5. Detection of bound antibody

The dilution of primary antibody was prepared in 5% BSA TBST washing buffer. For rabbit anti-phospho-Ser9-GSK-3 β or rabbit anti-total-GSK-3 β polyclonal antibody or mouse anti beta actin monoclonal antibodies at 1:1000 were used. For mouse anti-FLAG monoclonal antibody (1:2,000), rabbit anti cleaved caspase-8 polyclonal antibody (1:200), rabbit anti cleaved caspase-9 polyclonal antibody (1:1,000), cytochrome c (1:100), rabbit anti bax monoclonal antibody (1:2,000), mouse anti Cox IV monoclonal antibody (1:1,000), APP (1:1,000). Then the membrane was placed in clean tray containing primary antibody solution and incubated overnight at 4 $^{\circ}$ c. After incubation the primary antibody solution was discarded. Washing the membrane was performed with 20 ml of TBST in the period of 5 min for 3 times. The secondary antibody was prepared by TBST with 5% BSA. For β -actin, FLAG and Cox IV antibodies use goat anti mouse IgG (H+L) horseradish peroxidase (HRP) conjugate at 1 : 3,000. For GSK-3 β , caspase-8, caspase-9 and Bax antibodies use goat anti rabbit IgG (H+L) HRP conjugate at 1: 3,000. The membrane from the final wash in TBS was incubated for 1 h at room temperature with gentle agitation on a shaker. After that, wash the blot with 3 times of TBST for 5 min with gentle agitation on a shaker.

6. Protein detection

Supersignal west pico chemiluminescent substrate; prepare working solution by mixing equal parts of the stable peroxide solution and the luminol/enhancer solution. In a dark room, washed membrane was drained of excess buffer from and place in a fresh container. Then the detection reagent was directly added to the

membrane on the surface carrying the protein; do not leave the membrane to dry out. After incubating for precisely one min at room temperature, the excess detection reagent was drained off and the membrane was gently placed, protein side up, on a piece of Saran Wrap. A piece of Saran Wrap was placed over the membrane, smoothing out any creases or bubbles that may develop between membrane and Saran Wrap. It is necessary to work quickly once the membrane has been exposed to the detection solution. The membrane was placed, protein side up, in X-ray film cassette. The lights were turned off and sheet of autoradiography film carefully placed on the top of the membrane, the cassette was closed and exposed for 1 min (this depends on the amount of target protein on the membrane).

7. Buffers preparations for western blot

1.5 M Tris base (pH 8.8) 100 ml

Tris base	18.171	g
dH ₂ O	80	ml

Adjust the pH to 8.8 with conc. HCl and conc. NaOH

Adjust the volume to 100 ml with dH₂O

1 M Tris base (pH 6.8) 100 ml

Tris base	12.14	g
dH ₂ O	80	ml

Adjust the pH to 6.8 with conc. HCl and conc. NaOH

Adjust the volume to 100 ml with dH₂O

0.5 M Tris-HCl 100 ml

Tris base	6	g
dH ₂ O	40	ml

Adjust the pH to 6.8 with conc. HCl

Adjust the volume to 100 ml with dH₂O

10% SDS 100 ml

SDS	10	g
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Adjust the volume to 100 ml with dH₂O

1x Triton lysis buffer 100 ml

25 mM Tris-HCl (pH 8.0)	0.4	g
150 mM NaCl	0.88	g
0.5% Triton X-100	0.5	ml
5 mM EDTA	0.186	g
dH ₂ O	100	ml

4x reducing buffer 10 ml

62.5 mM Tris-HCl (pH 6.8), 0.5 M Tris-HCl (pH 6.8)	5	ml
8% (w/v) SDS, (SDS)	0.8	g
40% (v/v) glycerol, (glycerol)	4	ml
0.005% bromophenol blue, bromophenol blue	0.5	mg
Betamercaptoetanol	1	ml

5x Laemmli running buffer 1 L (for nitrocellulose membrane)

Tris base	15.1	g
Glycine	94	g
SDS	5	g

Adjust the volume to 1 L with dH₂O

1x Transfer buffer 4 L (for nitrocellulose membrane)

Tris base	12	g
Glycine	57.6	g
100% Methanol	800	ml
Adjust the volume to 4 L with dH ₂ O		

10x TBST washing buffer (pH 7.5) 3 L (for nitrocellulose membrane)

Tris base	363	g
NaCl	270	g
Adjust the volume with dH ₂ O		
Adjust the pH to 7.5 with conc. HCl		
Adjust the volume to 3 L with dH ₂ O		
Tween 20	30	ml

10x Laemmli running buffer (pH 8.3) 1 liter (for PVDF membrane)

Tris base	30.3	g
Glycine	144.2	g
SDS	10	g
dH ₂ O	900	ml
Adjust the pH to 8.3 with conc. HCl and conc. NaOH		
Adjust the volume to 1 liter with dH ₂ O		

10x Transfer buffer 1 liter (for PVDF membrane)

Tris base	5.8	g
Glycine	2.9	g
SDS	0.37	g
Adjust the volume to 800 ml with dH ₂ O		
100% Methanol	200	ml

10x TBS washing buffer (pH 7.4) 1 liter (for PVDF membrane)

Tris base	30	g
NaCl	80	g
KCl	2	g
dH ₂ O	800	ml

Adjust the pH to 7.4 with conc. HCl

Adjust the volume to 1 liter with dH₂O

Blocking buffer

5% BAS in TBST

Stripping buffer

100 mM Beta mercaptoethanol (5ml)

62.5 mM Tris-HCl Ph 6.8

2%SDS

Stripping buffer 10ml

Beta mercaptoethanol	79	μl
0.5 M Tris-HCl Ph 6.8	1.24	ml
SDS	2	ml

Adjust the volume to 10 ml with dH₂O

Stain 0.5% coomassei blue 500 ml

Methanol	200	ml
Acetic acid	50	ml
coomassei blue	2.5	g

Adjust the volume to 500 ml with dH₂O

Destain 500 ml

Methanol 150 ml

Acetic acid 50 ml

Adjust the volume to 500 ml with dH₂O

APPENDIX D

ZYMOGRAM AND SPECIFIC BUFFER

Table 10 Preparing the solutions for Tris/Glycine SDS-Polyacrylamide Gel Electrophoresis for zymogram

Reagents	10%Separating gel (ml)	4%Stacking gel (ml)
H ₂ O	4.1	3
1.5 M Tris-HCl (pH 8.8)	2.5	-
0.5 M Tris-HCl (pH)	-	2.5
Gelatin/Casein	0.01 (g)	0.01 (g)
10%SDS	0.1	0.05
30% Acrylamide mix	3.25	0.7
10% APS	0.05	0.025
TMED	0.004	0.004
Total Volume	10	5

1. Renaturing protein and developing protein

After running, Washed the gel with 3 changes of 1x renaturing buffer for 15 min each. Decant the zymogram renaturing buffer and replace with 1x zymogram developing buffer. Equilibrate the gel for 30 min at room temperature with gentle agitation then replace with fresh 1x zymogram developing buffer and incubate at 37°C overnight for maximum sensitivity. Stain gel with Coomassie Blue R-250 for 30 min. Areas of protease activity will appear as clear bands against a dark blue background where the protease has digested the substrate.

2. Buffer preparations for zymogram

2x non reducing sample buffer 10 ml

0.5 M Tris-HCl, pH 6.8	1.5	ml
Glycerol	2.5	ml
10% (w/v) SDS	4	ml
1% Bromophenol blue	0.1	ml
dH ₂ O	2.15	ml

5x Zymogram running buffer (pH 8.3) 600 ml

Tris base	9	g
Glycine	43.2	g
SDS	3	g
dH ₂ O	500	ml

Adjust the pH to 8.3 with conc. HCl and conc. NaOH

Adjust the volume to 600 sml with dH₂O

10x Renaturing buffer 100 ml

Triton X-100	25	ml
dH ₂ O	100	ml

10x Developing buffer 1 liter

1 M Tris base	12.1	g
Tris-HCl	63	g
NaCl	117	g
50 mM CaCl ₂	7.4	g
Brij-35	2	g
dH ₂ O	1000	ml

Stain 0.5% coomassei blue 500 ml

Methanol	200	ml
Acetic acid	50	ml
coomassei blue	2.5	g

Adjust the volume to 500 ml with dH₂O

Destain 500 ml

Methanol	25	ml
Acetic acid	37.5	ml

Adjust the volume to 500 ml with dH₂O

BIOGRAPHY

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