3. Methodology

Materials

$A\beta_{1-42}$ was purchased from American Peptide. TRI reagent, 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP), Insulin, progesterone, transferrin, NGF, poly-D-lysine, putrescine selenium In situ cell death detection kit, Rapamycin, and 3-Methyl adenine were purchased from Sigma (St. Louis,MO, USA). Anti-FoxO1, cleaved anti-LC3, anti-Akt, anti-p-Akt(Ser473) anti-p-mTOR (S2448), anti-mTOR, anti-p-ULK1 (Ser757), active Caspase-3, PARP and anti-ULK1 antibodies were from Cell Signalling Technology (Denver, MA, USA). Anti-LC3 antibody, Anti-Beclin1 antibody, Atg5 (Novus), anti-Trib3 antibody, pan-caspase inhibitor, Caspase3 inhibitor, Caspase8 inhibitor, Rapamycin, 3Methyl Adenine and LY 294002 were purchased from Calbiochem, (USA), anti-p62 antibody was purchased from R&D Systems (Minneapolis, MN,USA), anti-Bim and anti-Aβ antibodies were from Abcam (Cambridge, UK). Protein A agarose and HRP conjugated secondary antibodies were from Santa Cruz Biotechnology (Dallas, Texas, USA). Lipofectamine 2000, Alexa Fluor 488, Alexa Fluor 568, culture media and serum were purchased from Invitrogen, (Life technologies, Grand Island, NY, USA). PSD-95 antibody was obtained from Neuromab (UC Davis, Davis, CA, USA). Mitotracker Red was from Thermo Fisher Scientific (Waltham, MA, USA). Brain tissues of APPswe-PS1de9 mice and control littermates were kindly gifted by Dr. Anant B Patel (CSIR-CCMB, Hyderabad). About 12 months old male mice were used for the study.

Cell Culture

Cortical neurons from the neocortex of E18 day embryonic rat brains were cultured as described previously (1,2). The cells were plated on poly-D-lysine-coated culture plates and maintained in DMEM/F12 medium supplemented with insulin (25 µg/ml), glucose (6 mg/ml), transferrin (100 µg/ml), progesterone (20 ng/ml), putrescine (60 µg/ml) and selenium (30 ng/ml). Cultured neurons were subjected to treatment after 6 days. Primary hippocampal neurons were cultured from E18 rat hippocampus. The cells were plated on poly-D-Lysine-coated plates and cultured in neurobasal medium supplemented with B27 and maintained for three weeks. After three weeks the cells were used for treatment. Rat pheochromocytoma (PC12) cells were cultured as described previously (3) in RPMI medium supplemented with 10% heat-inactivated horse serum (HS) and 5% heat-inactivated fetal bovine serum. Neuronal differentiation

was induced by NGF (50 ng/ml) in medium containing 1% horse serum for 6 days before the treatment (4).

Preparation of Amyloid β

HPLC-purified $Aß_{1-42}$ was purchased from American Peptide (Sunnyvale, CA) and oligomeric $Aß_{1-42}$ was prepared (5). Lyophilized $Aß_{1-42}$ was reconstituted in HFIP to 1 mM, HFIP was then removed by evaporation in a Speed Vac, it was then resuspended to a concentration of 5 mM in anhydrous DMSO. This stock was then stored in -80^{o} C. The stock was further diluted with PBS to a final concentration of 400 μM and SDS was added to a final concentration of 0.2%. The resulting solution was incubated at 37^{o}C for 18-24 h. The preparation was finally diluted with PBS to a final concentration of 100 μM and incubated at 37^{o}C for 18-24 h before use.

Oligomeric Aβ treatment to cells

Oligomeric $Aβ_{1-42}$ was added to the medium containing cells for the specific time points. The concentrations used were, 1.5 μM for primary cultured rat cortical neurons and hippocampal neurons, and 5 μM for neuronally differentiated PC12 cells (6,7).

Intact nuclear counting assay

It was performed by using a detergent containing the buffer that dissolve only the cell membrane, leaving the nuclear membrane intact. The intact nuclei were then counted on a haemocytometer. The number of live cells was expressed as percentage of the total cell population.

PCR

Total RNA for each sample is isolated from cultured cortical neurons by using TRI reagent (Sigma). The primers used for PCR amplification of rat Trib3 were 5'-GTT GCG TCG ATT TGT CTT CA-3' and 5' -CGG GAG CTG AGT ATC TCT GG -3'. The primers for GAPDH were 5'-TCAACAGCAACTCCCACTCTT - 3' and 5'-ACCCTGTTGCTGTAGCCGTAT -3'. Equal amounts of cDNA template were used for each PCR analysis of Trib3 or GAPDH. Primers were used at 0.2 μM concentration. For semi-quantitative PCR, products were analysed on a 1.5% agarose gel and visualized by staining with ethidium bromide. Quantitative PCR were performed using

One Step SYBR *Ex Taq* qRT-Takara by using Applied Biosystems 7500 Fast Real Time PCR System following the manufacturer's specifications.

Western Blot Analysis

The cells were pelleted and lysed in appropriate amount of lysis buffer (10mM) Tris (pH 7.4),150mM NaCl, 1% TritonX100, 1mM EDTA, 1mM EGTA,0.2mM activated orthovanadate) on ice for 20 minutes. The lysates were centrifuged at12000rpm for 10 minutes. The supernatant was collected.The protein concentration of the sample was determined by the Bradford method, using BSA as standard. After protein estimation of the samples 50µg of the proteins were mixed with sample buffer (1X Tris.Cl/SDS pH.6.8, 30% glycerol, 10% SDS, 0.6M DTT and 0.012% bromophenolblue) and boiled for 4-5 minutes before loading. The proteins were boiled, spined and vortexed and loaded in the wells of the gel for SDS-PAGE. After the separation of the proteins in the gel the proteins were transferred from the gel to PVDF (Hybond: GE Healthcare, Buckinghamshire, UK) membrane by the wet type of transfer at 110 V for 90 minutes at 40C.After transfer, the PVDF membrane was stained with ponceau-S for 1 min to check proper transfer of the proteins from gel to membrane. The membrane was then washed with TBST (TBS-Tween20) until the red colour of Ponceau S disappeared. Then the membrane was blocked with 5% non fat dry milk
(NFDM) (Biorad) in 1X TBST for 1 hour at room temperature on a shaker. Then primary antibody was added and incubated for overnight at 40C (5% BSA in TBST was used as primary antibody diluent) on the dancing shaker. After washing the membrane for 3 times for 5 minutes with TBST the HRP conjugated secondary- antibody was added in appropriate dilution to the membranes and was kept for 1 hour on shaker. The membranes were washed with TBST 3 times for 5 minutes. After that the chemiluminescence assay was done by the milipore classic luminol reagent according to the manufacturer's protocol. Bands were detected on x-ray films (Kodak, Windsor, CO, USA).

Subcellular fractionation

Subcellular fractionation was done following Wang et al. (Wang Q et al 2006) with some modifications. In brief the cells were pelletted and resuspended in buffer A, containing 5mM HEPES, 210mM Mannitol, 70mM Sucrose, 0.5% BSA and a protease inhibitor cocktail. Four times volume by weight of buffer A was added to the cell pellet.

Cells were then lysed with 30–40 strokes in a homogenizer. Lysates were then centrifuged for 12 min at 500 rcf at 4^{o} C in a microcentrifuge. The supernatant is taken while the pellet is discarded. The pellet contains the crude nuclear fraction, which may also contain unbroken cells. The supernatant was further centrifuged 10,000 rcf for 10 min at 4^{o}C. The pellet contains the mitochondrial fraction, while the supernatant contains the cytoplasmic fraction. The pellet is resuspended in buffer A. Specific subcellular compartment markers were used to ensure the validity of this protocol.

Immunocytochemical staining

Cortical neurons and neuronally differentiated PC12 cells were fixed with 4% paraformaldehyde for 10 min (8,9). Cells were then washed thrice with PBS for 5 min each and then blocked in 3% goat serum in PBS containing 0.3% Triton-X 100 for 2 h at room temperature. The cells were immunolabelled with primary antibody in a blocking solution overnight at 4^{o}C.The next day cells were washed in PBS, followed by incubation with the appropriate secondary antibody for 2 h at room temperature. Nuclei were stained with Hoechst. High resolution images were taken using Leica TCS SP8 microscope (Germany).The intensities of staining for control or treated cells were quantified separately by NIH ImageJ software. The corrected total cell fluorescence (CTCF) was determined by considering the integrated density of staining, area of the cell and the background fluorescence for the different experimental conditions. CTCF = Integrated density – (area of selected cell × mean fluorescence of background readings).

TUNEL assay

Detection of TUNEL positive cells using in-situ cell death detection kit was performed following the manufacturer's protocol.

Mitochondrial staining

Cellular mitochondria were detected using Mitotracker red dye following the manufacturer's protocol.

Immunoprecipitation

Primary cultured cortical neurons were either treated with 1.5 μM Aβ for 8 h or left as untreated control and interactions of Trib3 with Akt were detected by co-

immunoprecipitation assays. The cells were washed with PBS and lysed in lysis buffer. For immunoprecipitation, agarose-conjugated anti-Trib3 antibody was prepared by incubating 3 μg anti-Trib3 antibody with 20 μl of protein A agarose beads for 2 h in 4°C under shaking condition. The agarose-conjugated Trib3 antibodies were then pelletted down and incubated with treated and untreated cell lysates containing equal amounts of protein overnight. This was kept at 4°C under shaking condition. The antigen–antibody complexes were isolated and dissociated by boiling in sample buffer for 4 min. The agarose beads were pelletted down and the supernatant was subjected to western blot analysis, as described previously (6).

RNA isolation

Total RNA was extracted using TRI reagent (Sigma). Cultured neuronal cells were homogenized in TRI reagent and centrifuged at 12,000 x g for 10mins at 4ºC to remove the insoluble materials such as high content of protein, fat, polysaccharides or extracellular materials. The supernatant was collected to a fresh tube and to ensure complete dissociation of nucleoprotein complexes, the sample was allowed to stand for 5 min at room temperature. 200μl of chloroform (per 1 ml of TRI) was added per sample and mixed by inversion by hand for 15 sec for phase separation. Next, sample was allowed to stand for 5-15 mins at room temperature and centrifuged at 12,000 x g for 15 min at 4ºC. The upper aqueous layer containing RNA was transferred carefully to freshly autoclaved microcentrifuge tubes. 500 μl of isopropanol (Merck) (per ml of TRI reagent) was added per tube, mixed and kept to stand at 5-10 min at room temperature and then centrifuged at 12,000 x g for 10 min at 4ºC. A distinct RNA pellet was formed. The pellet was washed with I ml of chilled 75% ethanol and vortexed and centrifuged again at 7,500 x g for 5 min at 4ºC. The resultant pellet is briefly

Chromatin immunoprecipitation (ChIP)

ChIP assays were done by using ChIP assay kit from Millipore (Billerica, MA, USA) following manufacturer's protocol with few exceptions. $5–8\times10^6$ cortical neurons were used after treatment with or without Aβ. Rabbit polyclonal anti-FoxO1 antibody was used to immunoprecipitate the protein–DNA complexes. The primers used for PCR amplification of the rat Trib3 promoter were forward 5'GTGCTGGGACTCCGAG

ATAG-3’ and reverse: 5’CAACCTTCTTG CCAGACCTC-3’ (10). PCR products were analyzed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Plasmid isolation:

After transformation, cells were plated on the Luria agar medium. The cells successfully transformed formed colonies on the plate. From those colonies a single colony was taken and inoculated in 30 ml Luria broth. After overnight incubation plasmids were isolated from the broth using Hi-pure plasmid midi-preparation kit (Invitrogen) following manufacturer’s instruction. 10 ml equilibrium buffer was applied to the hi-pure midi column. The solution was allowed to drain by gravity flow. The cells were collected from the overnight LB culture by centrifuging at 4000x g for 10 minutes. The entire medium was discarded. The cell pellet was homogeneously resuspended by adding 4 ml of resuspension buffer with RNaseA. Then 4ml lysis buffer was added to the suspension and was mixed gently by inverting (without vortexing). Then the mixture was incubated at RT for 5 min. 4 ml precipitation buffer was added to the mixture followed by immediate inversion of the tube until the mixture was homogeneous. Then it was centrifuged at >12000x g for 10 minutes at RT. Then the supernatant was loaded on the equilibrated column with a pipette. The solution was allowed to go through the column to drain by gravity flow. Then column was washed twice with 10 ml wash buffer. The flow through was discarded. Then 15 ml tube was placed under the column and 5ml elution buffer was added to the column and was collected in 15 ml tube, it contains purified DNA. Then 3.5 ml of isopropanol was added to elute and the solution was centrifuged at >12000x g for 30 minutes at 40c. Then the pellet was collected and 3 ml 70% ethanol was added and it was again centrifuged at >12000x g for 5 minutes at 40C. Supernatant was discarded. The pellet was air dried for 10 min. Then to that pellet 100 to 200µl of TE buffer was added and plasmid DNA was redissolved. It was kept at -20^0C.

Transfection

DNA was isolated using Plasmid Maxi kit from Qiagen. For survival assay, cortical, hippocampal neurons and PC12 cells were transfected with 0.5 µg of either pSIREN-Trib3-shRNA-zsgreen (shTrib3) or pSIREN-Rand-shRNA-zsgreen (shRand), pWPI plasmid containing Trib3 overexpression vector and the corresponding control plasmid.

Transfections were done in 500 µl of serum-free medium per well of a 24-well plate using lipofectamine 2000. Five hours later, lipofectamine containing medium was replaced by fresh medium. Transfection was performed on the third day of culture for primary cortical neurons and on the nineteenth day of culture for primary hippocampal neurons. Differentiated neuronal PC12 cells were transfected on the third day of differentiation. For endogenous Trib3 downregulation, naive PC12 cells were transfected with either 1µg of shTrib3 or shRand. Transfections were done in 1ml of serum-free medium per well of a 12-well plate using lipofectamine 2000. 24 h post-transfection cells were differentiated in presence of NGF. After 5 days of priming, cells were treated with either Aβ (5µM) or left as untreated control.

Sholl analysis

Sholl analysis was performed to analyse the neuritic branching and complexities of neuronal processes using NIH-ImageJ software as described previously (11) with a few modifications delineated below. Primary hippocampal neurons (19 DIV) were transfected with shFoxO or shRand as described previously. A fluorescence microscope was used to image the transfected neurons. Low-magnification pictures of single neurons were taken at 0 h and 48 h of $A\beta_{1-42}$ treatment. Sholl analysis was performed on these images using NIH-ImageJ software (Sholl analysis plugin). A number of concentric circles were drawn projecting from the cell body with gradually increasing radii of 40 mm in length. Two dimensional analyses were performed to count the number of branches that intersect the successive concentric circle. Data are represented as mean ± S.E.M. of four neurons from three independent experiments.

Survival assay

Primary cortical neurons (3DIV) and primary hippocampal neurons (22 DIV) were transfected with shRand or shTrib3 as mentioned above. 48 h post transfection, the neurons were exposed to $A\beta_{1-42}$ and the number of transfected neurons (green) was counted (0 h) under the microscope. The number of surviving transfected neurons were also counted after 24 h, 48 h and 72 h of treatment as described previously (4). Control and Aβ-treated transfected neurons were imaged under a fluorescence microscope (Leica, Wetzlar, Germany). Data are represented as mean ± S.E.M. of three independent experiments which were performed in triplicate.

Oligomeric Aβ infusion in animals

Five Male Sprague-Dawley rats (300–380 g) were infused with oligomeric Aβ as described previously, five male rats were infused with PBS. Briefly, rats were anaesthesized by injecting a mixture of xylazine–ketamine, placed on a stereotaxic frame. A volume of 5µl of 100 µM Aβ in PBS was infused in the right cerebral cortex at stereotaxic co-ordinates from bregma: AP: – 4.1, L: 2.5, DV: 1.3 mm, according to the rat brain atlas and a previous report (12). Equal volume of PBS was injected in control animals. Animals were sacrificed 21 days post injection. The brains were dissected out following cardiac perfusion and fixed in 4% PFA for 24 h. The brains were further incubated in a 30% sucrose solution for 24 h and then cryo sectioning was performed by using a cryotome (Thermo, West Palm Beach, FL, USA).

Immunohistochemistry of brain slices

Cryo sections measuring twenty micrometre of the brains from Aβ-infused or PBS-infused rats and wild-type or transgenic mice were immunostained as described previously. Briefly, sections were blocked with 5% goat serum in PBS containing 0.3% Triton-X 100 for 1h at room temperature; sections were incubated in primary antibody in a blocking solution overnight at 4˚C. The following day the sections were washed with PBS thrice and then incubated with a fluorescence-tagged secondary antibody for 2 h at room temperature. Hoechst staining for the nucleus was performed. The sections were mounted and observed under fluorescence microscope.

shRNA Constructs

Trib3 shRNA, shFoxO and shRAND were described previously (6,10). Beclin shRNAs were prepared in the pSIREN vector by using BD Knockout RNAi systems (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions based on the sequences- 5'-GATCCCATCCATCGGCTTCATATTCAAGAGATATGAAGCCGATGGAACTGTTTTTTG-3' and 5'-AATTCAAAAAACAGTTCCATCGGCTTCATATCTCTTGAATATGAAGCCGATGGAACTGG-3'. shRNA were transfected using Lipofectamine 2000 at a final level of 0.5 ug per 24 well culture.

Statistics

All experimental results are reported as mean ± S.E.M. Student's t-test was performed as unpaired, two-tailed sets of arrays to evaluate the significance of difference between the means and are presented as p-values.

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