4. MATERIALS AND METHODS

4.1 Cells and reagents

SH-SY5Y cells were grown at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco, Invitrogen) supplemented with 15% (v/v) heat-inactivated fetal bovine serum (Gibco, Invitrogen) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Gibco, Invitrogen). GFP-LC3 and mCherry-p62-GFP were provided by Dr. T. Yoshimori (National Institute of Genetics, Shizuoka, Japan) (Kabeya *et al.*, 2000). α-Synuclein-GFP was provided by Dr. Patrik Brundin (Neuronal Survival Unit, Wallenberg Neuroscience Center, Sweden). The primary antibodies used were: mouse monoclonal against caspase-9 (Cell Signaling, USA), rabbit polyclonal against VCP (Cell Signaling, USA), rabbit polyclonal against caspase-3 (Cell Signaling, USA), PARP (Cell Signaling, USA), LC3 (Sigma, USA) and β- actin (Abcam, USA). Secondary antibodies: HRP-conjugated anti-rabbit and anti-mouse antibodies (Open Biosystems, USA) were used in the study. Rotenone, H_2O_2, 6-OHDA, N-acetyl-cysteine (NAC), Antimycin A, Sodium azide, Decylubiquinone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Acridine orange and Propidium Iodide (PI) were purchased from Sigma-Aldrich, USA. TNF-α was purchased from Enzo Life Science (USA), z-VED.fmk from Invivogen (USA), SYBR green from Takara (Japan), first strand cDNA kit, miRCURY miRNA isolation kit from Exiqon (Denmark), miR-5701 mimic, Anti-miR-155 from Ambion (USA), Tetramethylrhodamine, methyl ester (TMRM) and ATP assay kit from Invitrogen (USA).

4.2 Cell death assay

MTT assay: The cell viability was analyzed by MTT assay and Acridine/Propidium Iodide staining. SH-SY5Y cells were plated at the density of $1X10^4$ cells/per well in 96 well plate. After 24 hours of culture, the cells were treated with indicated chemicals. MTT assay was performed at specified time intervals by incubating cells with MTT (0.1mg/ml) at 37°C for 1-2 hours. The visible blue crystals were solubilized in DMSO and the absorbance was measured at 510 nm using microplate reader μQuant (Biotech Instruments, USA).

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Acridine/Propidium Iodide staining: The cells were plated in 24 well plate at the density of 1.5 X 10^5 cells/well. The cells were treated with indicated chemical and for indicated time. The Acridine orange/Propidium iodide (0.025 µg/ml and 0.033 µM respectively) solution was added to cells, plates were centrifuged and images were captured using GFP and RFP filter by 1X81 fluorescent microscope (Olympus, Japan). Images were captured in monochrome and pseudo-coloured and analysed for green (healthy), orange (apoptotic) and red cells (late apoptotic or necrotic) using Image-Pro Plus (Media Cybernetics, USA). Caspase3/7 activation assay was performed using Caspase-Glo® 3/7 Assay kit (Promega, USA), according to manufacturer protocol.

4.3 Western blotting

SH-SY5Y cells were plated in 6 well plate at the density of 5 X 10^5 cells/well. The cells were treated with indicated chemical and for indicated time. Cells were harvested, washed with ice cold PBS and lysed in NP40 lysis buffer (150mM NaCl, 50mM Tris-Cl, 1% NP40, 1mM PMSF). Protein concentration was determined by Bradford assay and equal protein loaded on 12% SDS-PAGE. Protein was electroblotted on PVDF membrane at 110V for one hour. Following the transfer, the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 hour at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation the membrane was washed three times with TBS-T (TBS containing 0.1% Tween 20) for 10 minutes and incubated with a secondary antibody at room temperature for 1 hour. The membrane was similarly washed three times with TBS-T and protein bands on the membrane were then visualized by using EZ-ECL chemiluminescence detection kit for HRP (Invitrogen, USA) by exposing to X-ray film.

4.4 Generation of stable cell line

The stable cell line SH-SY5Y-MTRFP was generated to study the mitophagy and mitochondrial dynamic. To generate SH-SY5Y-MTRFP stable cell line, 1.5×10^5 cells of SH-SY5Y were plated in 24 well plate and transfected with mitochondrial targeted red fluorescent protein vector (pHcRed1-Mito) (Clonetech, USA) using FUGENE HD

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Transfection Reagent (Roche, Germany) according to manufacturer's protocol. Media was replaced with DMEM-F12 supplemented with G418 (500 μg/ml) after 48 hrs of transfection and every alternate day until stable clones were clearly visible. The stable cells were harvested from 24 well plate and transferred to 96 well plate to obtain single clone using serial dilution method. The single clones were transferred and maintained in 6 well plate. After incubation for 15 days, the cells were transferred to 25 cm^2 culture flask and maintained in DMEM-F12 supplemented with 50 μg/ml of G418.

4.5 Analysis of mitophagy by confocal microscopy

SH-SY5Y-MTRFP cells were plated at the density of 1.5 X 10^5 cells per well in 24 well plate having coverslip and transfected with GFP-LC3 using FuGENE HD Transfection Reagent (Roche, Germany). After 24 hours of transfection, the cells were treated with 20 ng/ml of TNF-α for 72 hours and co-localization of GFP-LC3 with mitochondria was monitored using Leica TCS-SP5-II confocal microscope (Germany).

SH-SY5Y cells were plated at the density of 1.5 X 10^5 cells per well in 24 well plate having coverslip and co-transfected with mcherry-p62-GFP and miR-5701 mimic using Lipofectamine® RNAiMAX (Thermofisher, USA). After 24 hrs of transfection, autophagy flux was monitored using Leica TCS-SP5-II confocal microscope (Germany).

4.6 Analysis of reactive oxygen species and mitochondrial membrane potential

ROS was analysed by microscopy and spectrofluorometry using MitoSOX as mitochondria ROS indicator. SH-SY5Y cells were treated with indicated chemicals. After incubation for indicated time, the cells were stained with MitoSOX Red (1.0 μM) (Invitrogen, USA) in DMEM-F12 for 10 min at 37 °C. Cells were washed three times in fresh DMEM-F12. Images were captured using 10X objective (Olympus IX81 microscope) having same exposure for each well.

The relative quantification of ROS was analyzed by spectrofluorometry. Cells were harvested after treatment as indicated. Cells were suspended in DPBS containing MitoSOX Red (1.0 μM) (Invitrogen, USA) for 10 min. The cells were washed with

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DPBS and fluorescence was analyzed by fluorescence spectrophotometer (Hitachi, Japan) at Excitation 510 and Emission 580.

Mitochondrial membrane potential (ΔΨ) was analyse by spectrofluorometry using TMRM staining. SH-SY5Y cells were loaded with TMRM (200 nM) (Invitrogen, USA) for 15 min after treatment with indicated chemicals. The cells were washed in DPBS and fluorescence was analyzed by fluorescence spectrophotometer (Hitachi, Japan) at Excitation 540 and Emission 570.

4.7 Analysis of complex-I activity

Complex-I activity was determined by BN-PAGE (Blue-Native Page) and Spectro-Photometery. BN-PAGE was performed as described previously (Yan *et al.*, 2007; Yan and Forster, 2009) with minor modifications. Acrylamide (50% w/v)/Bisacylamide (0.5% w/v) solution was prepared such that it yielded highly porous gels on polymerization, for the separation of higher order protein complexes. The final concentration of the stacking gel was 4% and that of the resolving gel was 10%. Purified mitochondrial fraction was mixed with sample buffer and non-gradient BN-PAGE was performed at room temperature as described previously (Yan and Forster, 2009). The gel was stained with complex-I staining solution (50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mg/ml NBT and 0.1 mg/ml NADH). All gel images were documented using an Epson Perfection 1670 scanner.

The activity of complex-I was determined using decylubiquinone as the electrons acceptor and NADH as donor. Briefly, cells were harvested and washed with cold DPBS. Cells were subjected to freeze-thawing two or three times in Freeze-Thaw complete solution (0.25M Sucrose, 20mM Tris-HCl (pH 7.4), 40 mM KCl, 2mM EDTA supplemented with 1mg/ml fatty acid free BSA, 0.01% Digitonin and 10% Percoll). After completion of the freeze-thawing process, the cells were washed with Freeze-Thaw solution devoid of digitonin to remove the residual digitonin as it interferes with the complex-I enzyme assay. Cells were suspended in complex-I assay buffer (35mM Potassium Phosphate (pH-7.4), 1mM EDTA. 2.5 mM NaN3, 1mg/ml BSA, 2µg/ml

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Antimycin A, 5mM NADH). Complex-I activity was measured for 3 min by following the decrease in absorbance at 340nm after the addition of 2.5 mM acceptor decylubiquinone indicating the oxidation of NADH.

4.8 ATP luciferase assay

Total and mitochondrial ATP was performed by using ATP Bioluminescence kit CLS II (Invitrogen, USA). Briefly, SH-SY5Y cells were treated with indicated chemicals for indicated time. Mitochondrial fraction was isolated by differential centrifugation method (Schug *et al.,* 2011). Total cell and mitochondrial fractions were lyszed in 0.1 % TritonX-100 and ATP levels were analyzed using Luminescent ATP Detection Assay Kit (Invitrogen, USA) according to the manufacturer's instructions. ATP concentration was calculated by plotting standard curve.

4.9 miRNA isolation and analysis by quantitative Real Time-PCR (qRT-PCR)

SH-SY5Y cells were plated at density of 5 X 10^5 cells/well in 6 well plate. The cells were treated with indicated chemical and for indicated time. miRNAs were isolated using miRCURY miRNA isolation kit (Exiqon, Denmark) according to manufacturer protocol.

The expression of miRNAs was determined by qRT-PCR. For analysis of miRNA expression, Poly-A tailing of 100 ng miRNA was performed at 42°C for 1 hour using PolyA polymerase (NEB, England). The cDNA was synthesized using universal degenerate primer and Universal RT mix (Applied Biosystems, USA). qRT-PCR was performed with SYBR Premix Ex Taq II (Takara, Japan) with StepOnePlusTM (Applied Biosystem, USA) for 35 cycles of 95 °C for 15 sec and 60 °C for 60 sec. 5S rRNA was used as endogenous control.

The target mRNA levels were also analyzed by qRT-PCR. Briefly, total RNA was isolated using Trizole Reagent (Life technologies, USA) and was reverse transcribed to synthesize cDNA using cDNA Universal RT mix (Applied Biosystems, USA) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Premix Ex TaqTM (Takara, Japan) as per the manufacturer's instructions for 35 cycles of 95 °C for

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5 sec and 60 °C for 34 sec. GAPDH was used as endogenous control. Optimal dilution and melting curves were utilised for specificity of amplified production for each primer set. The relative amount of each gene and miRNA was measured using the 2(-ΔΔCT) method (Livak and Schmittgen, 2001). The relative association with standard error was plotted using Graph Pad PRISM 5.0.

Specific primers of the genes are listed below.

1. ND1: Fwd 5′- ATACCCCCGATTCCGCTACGAC -3′;

   Rev, 5′-GTTTGAGGGGGAATGCTGGAGA -3′;

2. ND2: Fwd 5′-ATTCCATCCACCCTCCTCTC -3′;

   Rev 5′-TGGGGTGGGTTTTGTATGTT -3′;

3. ND3: Fwd 5′-CCCTCCTTTTACCCCTACCA-3′

   Rev 5′-GGCCAGACTTAGGGCTAGGA -3′;

4. ND4L: Fwd 5′-TAACCCTCAACACCCACTCC -3′;

   Rev 5′-GGCCATATGTGTTGGAGATTG -3′;

5. ND5: Fwd 5’-CAAAACCTGCCCCTACTCCT-3′;

   Rev 5′-GGGTTGAGGTGATGATGGAG -3′

6. ND6: Fwd 5’- GGGTGGTGGTTGTGGTAAAC -3′;

   Rev 5′ -CCCCGAGCAATCTCAATTAC- 3′

7. ATP6: Fwd 5′- CGCCACCCTAGCAATATCAA -3′;

   Rev, 5′-TTAAGGCGACAGCGATTTCT-3′;

8. ATP8: Fwd 5′-ATGGCCCACCATAATTACCC -3′;

   Rev 5′-GCAATGAATGAAGCGAACAG -3′;

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9. COX1: Fwd 5′-CGATGCATACACCACATGAA -3′

Rev 5′-AGCGAAGGCTTCTCAAATCA -3′;

10. COX2: Fwd 5′-TGAAGCCCCCATTCGTATAA -3′;

Rev 5′-ACGGGCCCTATTTCAAAGAT-3′;

11. COX3: Fwd 5’-GGCATCTACGGCTCAACATT -3′;

Rev 5′-CGAAGCCAAAGTGATGTTTG -3′

12. ND6: Fwd 5’- GGGTGGTGGTTGTGGTAAAC -3′;

Rev 5′ -CCCCGAGCAATCTCAATTAC- 3′

13. β-Actin: Fwd 5′-TCGTGCGTGACATTAAGGGG-3′

Rev 5′-GTACTTGCGCTCAGGAGGAG-3′;

14. 16s rRNA: Fwd 5′-GAAACCAGACGAGCTACCTAAG-3′;

Rev 5′-GCCTCTACCTATAAATCTTCCC-3′;

15. GAPDH: Fwd 5’-AGAAGGCTGGGGCTCATTTG-3′;

Rev 5′AGGGGCCATCCACAGTCTTC 3′

16. PGC1: Fwd 5’- GAGCCGAGCTGAACAAGCAC-3′;

Rev 5′ -AGACACATTGAACAATGAATAGGATG - 3′

17. NRF1: Fwd 5’- GGCACTGTCTCACTTATCCAGGTT-3′;

Rev 5′ -CAGCCACGGCAGAATAATTCA- 3′

18. NRF-2: Fwd 5’- AAACCAGTGGATCTGCCAAC-3′;

Rev 5′ - GACCGGGAATATCAGGAACA -3′

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19. VCP: Fwd 5′- TGACAGAGATTTGCCAGCGT-3′;

Rev 5′- CCTCCATGGCTGATGGGTTT-3′;

20. Lysosomal protein transmembrane 4α:

Fwd 5’- GCCTGTGTTCTTTTTGCCGT-3′;

Rev 5′- GGCAACTGAGGACGAAGTCA- 3′

21. ATPase: Fwd 5’- TCTGGTGATGACTTGAAACTGC-3′;

Rev 5′ - GTCTAGGAAGCTGGCGAGTG -3′

22. mtDNA: Fwd 5’- CACTTTCCACACAGACATCA -3′;

Rev 5′ - TGGTTAGGCTGGTGTTAGGG -3′

23. RNAse P: Fwd 5’- CCCCGTTCTCTGGGAACTC -3′;

Rev 5′ - TGTATGAGACCACTCTTTCCCATA -3′

miRNAs Primer:

1. hsa-miR-4485: Fwd-AGUAACGGCCGCGGUAC

Rev-TCCAGTTTTTTTTTTTTTTTAGG

2. hsa-miR-23a: Fwd-CATCACATTGCCAGGGAT

Rev-CGTCCAGTTTTTTTTTTTTTTTGGAA

3. hsa-miR-23b: Fwd-CATCACATTGCCAGGGAT

Rev-TCCAGTTTTTTTTTTTTTTTGTGGTA

4. hsa-miR-103: Fwd-AGAGC AGC ATT GTA CAG GG

Rev-GTCCAGTTTTTTTTTTTTTTTCATAG

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5. let-7g: Fwd-GGCAGTGAGGTAGTAGT TTGT

Rev-GGTCCAGTTTTTTTTTTTTTTTAACTGTAC

6. hsa-miR-4792: Fwd-AGTTTTTTTTTTTTTTTTTGCCAGC

Rev-GCAGCGGTGAGCGCT

7. hsa-miR-4511: Fwd-AGTTTTTTTTTTTTTTTTTAGGGCAA

Rev-GCAGGAAGAACUGUUGCAU

8. hsa-miR-711: Fwd-CCAGTTTTTTTTTTTTTTTTTCTTACGT

Rev-GCAGGGGACCCAGGGAGA

9. hsa-miR-1287: Fwd-CAGTTTTTTTTTTTTTTTTTGACTCGA

Rev-GCAGTGCTGGATCAGTGGT

10. hsa-miR-22-3p: Fwd-GTCCAGTTTTTTTTTTTTTTTTTACAGTTC

Rev-GCAGAAGCTGCCAGTTGAA

11. hsa-miR-5701: Fwd-GTCCAGTTTTTTTTTTTTTTTTTAATCAG

Rev-GCAGGCAGTTATTGTCACGT

12. hsa-miR-181b-5p: Fwd-AGTTTTTTTTTTTTTTTTTACCCACC

Rev-GCAGAACATTCATTGCTGT

13. hsa-miR-125a-5p: Fwd-AGTTTTTTTTTTTTTTTTTTCACAGGTT

Rev-GCAGTCCCTGAGACCCTTTA

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14. hsa-miR-548as-3p: Fwd-TCCAGTTTTTTTTTTTTTTTTACAAAC

Rev-GCGCAGTAAAACCCACAATTAT

15. hsa-miR-1184: Fwd-AGTTTTTTTTTTTTTTTTTGGAAGCC

Rev-GCAGCCTGCAGCGACTTGAT

16. hsa-miR-34a-5p: Fwd-CCAGTTTTTTTTTTTTTTTTTACAACC

Rev-CGAGTGGCAGTGTCTTAGCT

17. hsa-miR-92a-3p: Fwd-CCAGTTTTTTTTTTTTTTTTTACAGGC

Rev-CGAGTATTGCACTTGTCCCG

18. hsa-miR-128a: Fwd-CAGTCACAGTGAACCGGT

Rev-CCAGTTTTTTTTTTTTTTTTTAAAGAG

19. hsa-miR-155: Fwd-GGCAGTTAATGCTAATCGTGA

Rev-AGTTTTTTTTTTTTTTTTTACCCCTA

20. hsa-miR-27a: Fwd-GGCAGTTCACAGTGGCTAA

Rev-GTTTTTTTTTTTTTTTTGCGGA

21. hsa-miR-16-5p: Fwd-GGCAGTAGCAGCACGTAAATA

Rev-GTTTTTTTTTTTTTTTTCGCCAA

22. hsa-miR-17-5p: Fwd-GCAGCAAAGTGCTTACAGTG

Rev-GCAGTTTTTTTTTTTTTTTCTACCT

23. hsa-miR-98-5p: Fwd-GCGCAGTGAGGTAGTAAGTTGT

Rev-GCAGTTTTTTTTTTTTTTTTAACAAT

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24. hsa-miR-204-3p: Fwd-CAGGCTGGGAAGGCA

Rev-AGTTTTTTTTTTTTTTTTTACGTCC

4.10 Microarray

SH-SY5Y cells were plated at the density of 5 X 10^5 cells/per well in 6 well plate. After 24 hrs of culture, the cells were treated with indicated chemicals for 24 hrs. The total cellular RNA was extracted using miRCURY™ RNA Isolation Kit (Prod. No.300110, Exiqon). The RNA integrity was determined using Agilent 2100 Bioanalyzer (Ambion, USA). The RNAs from both sample and reference were labeled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon, Denmark) following the procedure described by the manufacturer. The Hy3™-labeled samples and Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon, Denmark), which contained capture probes targeting all miRNAs for human, mouse or rat registered in the miRBASE 18.0. The quantified signals were background corrected (Normexp with offset value 10) and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. The experiment was performed in three independent biological replicates.

4.11 Analysis of reactive oxygen species and LysoSensor staining

SH-SY5Y cells were plated at the density of 1.5 X 10^5 cells per well in 24 well plate and transfected with miR-5701 mimic and control using Lipofectamine® RNAiMAX (Thermofisher, USA). After 24 hours of transfection, cells were treated with indicated chemical and for indicated time and ROS was check as described previously (Prajapati *et al.*, 2015).

LysoSensor staining was performed using LysoSensor™ Green DND-189 (Thermofisher, USA) as per manufacture protocol. Fluorescence intensity was measure using the fluorometer (Hitachi, Japan) at Excitation 440 and Emission 510.

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4.12 Biotin-tagged miRNA pull-down and target validation

The targets of miR-5701 were determined by biotin pull-down method of Orom et al. (Orom and Lund, 2007) with minor modifications. Briefly, SH-SY5Y cells, were transfected with synthetic biotin-labeled miR-5701 duplexes (custom designed from Exiqon, Denmark) using HiPerFect Transfection Reagent (QIAGEN, Melbourne, VIC, Australia) with negative control. After 24 hours, cells were harvested and lysed in hypotonic lysis buffer (10 mM KCl, 1.5 mM MgCl2,10 mM Tris-Cl pH 7.5, 5 mM DTT, 0.5% NP-40, 60U/ML SUPERase•In (Ambion, Austin, TX, USA) and 1X protease inhibitor (Roche, Brisbane, QLD, Australia). Cell lysate was centrifuged at 10,000 g at 4°C for 2 minutes and supernatant was collected and calibrated to 1M NaCl in fresh vial and further incubated with 25 µl of myOne C1 Dynabeads (Thermofisher, USA). The beads were washed and pre-blocked with 1 µg/µl bovine serum albumin and 1 µg/µl yeast tRNA (Invitrogen) prior of incubation. The beads were further washed thrice with hypotonic lysis buffer and 1 M NaCl and RNA extracted for target identification.

4.13 Statistical analysis

Data are shown as mean ± SEM for number of observations. Comparisons of groups were performed using student t-test for repeated measurements to determine the levels of significance for each group. Each experiment has been repeated minimum two times independently and probability values of $p<0.05$ were considered as statistically significant.

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