Chapter 3. Materials & Methods

3.1 Kits and reagents

U-87 MG, SK-N-SH, and SH-SY5Y cell lines were sourced from the National Centre for Cell Sciences in Pune, India. One-shot Exosome-depleted FBS was obtained from Gibco, Invitrogen. Dr. Terje Johansen from the Department of Biochemistry, Institute of Medical Biology, University of Tromsø, generously supplied the mCherry-GFP-LC3.

The primary antibodies used included Anti-LC3 (Sigma, USA), Anti-p62 (Cell Signalling, USA), Anti-NDP52 (Cell Signalling, USA), Anti-PARP (Cell Signalling, USA), Anti-caspase-3 (Cell Signalling, USA), Anti-CD63 (SantaCruz, USA), Anti-Actin (GenScript, USA), and Anti-Calnexin (Cell Signalling, USA). Secondary antibodies, namely HRP-conjugated anti-rabbit, and anti-mouse antibodies (Jackson ImmunoResearch, USA) were employed.

Rotenone and 6-OHDA were acquired from Sigma-Aldrich, USA, along with Bafilomycin A1, rapamycin and wortmannin. Transfection in cells was done by Lipofectamine® 3000 from Invitrogen, USA. MTT, CM-H2DCFDA, and TMRM were purchased from Thermo Fisher, USA. SYBR (TB Green Premix Ex Taq II) and a cDNA synthesis kit (PrimeScript 1st strand cDNA Synthesis Kit) were obtained from Takara, Japan. For droplet digital PCR, the reagents were sourced from Bio-Rad, USA.

3.2 Cell culture and seeding density

SH-SY5Y, U-87 MG, and SK-N-SH cells were cultured at 37°C with 5% CO2 in MEM/EBSS (Hyclone, GE Lifesciences) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen), 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Gibco, Invitrogen), and 1mM Sodium Pyruvate (Hyclone, GE Lifesciences).

3. 3 Isolation of primary neurons and seeding density

Primary neuron cultures were established following the procedure outlined by Volpicelli-Daley et al. [267]. To summarize, embryonic E17 brains were extracted from pregnant Sprague-Dawley rats using methods approved by the Purdue Animal Care and Use Committee. The cortex and

mesencephalic region, which includes the substantia nigra and ventral tegmental area, were carefully isolated under stereoscopic guidance.

The tissue was subjected to papain treatment (20 units/mL in 1X HBSS) and then vigorously pipetted to create a dissociated cell suspension. This cell suspension was plated on plates coated with poly-D-Lysine in a culture medium comprising Neurobasal, 2% B-27 supplement, 5% fetal bovine serum (FBS), Glutamax, and 1% (w/v) Pen-Strep. The following day, the plating medium was replaced with complete Neurobasal medium supplemented with B-27 and Glutamax, but without FBS. The neurons were maintained in this medium for a minimum of 5 days before the initiation of any treatments.

3.4 Treatment of PD-stress inducers

SH-SY5Y dopaminergic neuronal cells and U-87 MG glial cells were exposed to a treatment consisting of 75 μM 6-OHDA and 0.01 μM rotenone, while SK-N-SH dopaminergic neuronal cells received treatment with 5 μM 6-OHDA and 0.1 μM rotenone.

Primary midbrain neurons and primary cortical neurons received 25nM and 60nM of rotenone, respectively.

3.5 Rotenone rat model

Sprague-Dawley rats were bred in-house for the study. Male rats aged 3 months (young adult) were used for the study. All the rats were housed under standard 12h light cycle, with free access to water and fed *ad libitum*. All the methods used in the study were approved by the Purdue Animal Care and Use Committee. Rats were divided into 3 following groups: 11 treated with vehicle (V), 10 treated with rotenone for 8h (R8), and 11 treated with rotenone for 24h (R24). The rotenone dose (3mg/kg) and acute timepoints were chosen to capture preclinical changes prior to cell loss and behavioural phenotypes [268](69).

Rotenone solution was prepared fresh in 98% Miglyol 812N and 2% DMSO (dimethylsulfoxide), which on vortexing, produces a stable emulsion of DMSO containing Miglyol 812N and rotenone. The rotenone was administered to the rats intraperitoneally and sacrificed 8h or 24h after

injections. Two sets of injections were given to each rat, where R8 and R24 received one injection with only vehicle, and another containing rotenone, and V received both injections containing vehicle solution.

Rats were anaesthetized by isoflurane gas, after which CSF collection was performed from the cisterna magna by direct puncture as described in the protocol (55). After CSF collection, blood from rats was collected using cardiac puncture. Post-euthanasia, the brain was rapidly removed, and brain regions, like, dorsal and ventral striatum, dorsal and ventral midbrain, cerebral cortex, and hippocampus were dissected on ice, flash frozen and then stored in -80ºC for processing of miRNA analysis.

3.6 Exosome isolation: from cells, serum, and cerebrospinal fluid (CSF)

Cells were initially plated and were exposed to pre-conditioned media containing serum that had been depleted of exosomes. The exosomes were isolated using an affinity purification-based method. The pre-conditioned media was collected and then subjected to centrifugation at 2000 g for 30 minutes to allow cell debris to settle down. The resulting supernatant was collected, and Total Exosome Isolation reagent from Thermo Fisher Scientific (Invitrogen, USA) was added in a 2:1 volume ratio. This mixture was left to incubate at 4°C overnight. Following incubation, the mixture was centrifuged at 10,000 g for 1 hour at 4°C to recover the exosome pellet.

In the case of CSF, the initial step involved centrifuging the cerebrospinal fluid at 2000g for 30 minutes at 4ºC to eliminate cells and debris. Subsequently, the resulting supernatant was carefully collected and subjected to a further round of centrifugation at 10,000g for an additional 30 minutes at 4ºC. The clarified CSF, found in the supernatant, was then transferred to a fresh tube. To this, an equal volume of Total Exosome Isolation reagent (specially designed for body fluids) from ThermoFisher Scientific (Invitrogen, USA) was added. This mixture was thoroughly blended through pipetting until a uniform solution was achieved. The sample was left to incubate at 4ºC for 1 hour. After incubation, the samples underwent centrifugation at 10,000g for 1 hour at 4ºC. The supernatant was removed via pipetting, leaving the exosome pellet at the bottom of the tube, which was then further processed as required.

For serum, the process began with the extraction of crude serum from whole blood. The blood was allowed to coagulate on ice for 30 minutes, and then the samples were centrifuged at 2000g for 15 minutes at 4ºC to isolate the serum in the supernatant. This serum was subsequently used for exosome extraction. The serum was initially centrifuged at 2000g for 30 minutes at 4ºC to eliminate any cellular debris. The resulting supernatant was transferred to a new tube, and Total Exosome Isolation reagent designed for serum from ThermoFisher Scientific (Invitrogen, USA) was added in a 5:1 volume ratio. The mixture was thoroughly mixed until the solution became milky, and it was then incubated at 4ºC for 30 minutes. After the incubation period, the samples were centrifuged at 10,000g for 30 minutes at 4ºC to yield the exosome pellet.

3.7 Nanoparticle Tracking Analysis (NTA)

The exosome pellet obtained from the above-mentioned methods was reconstituted in 1 ml of PBS solution and subsequently examined using the NanoSight NS300 system from Malvern Panalytical, UK. This system provides data on both the size and concentration of the particles.

3.8 Western blotting

Following treatments, the samples were harvested, rinsed with ice-cold PBS, and then lysed using NP40 lysis buffer, which contained 150mM NaCl, 50mM Tris–Cl, 5mM EDTA, 1% NP40, 1% Glycerol, and 1× protease inhibitor cocktail from SIGMA (USA). To determine the protein concentration, a Bradford assay was performed using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, USA), and an equal amount of protein was loaded onto a 10.5% SDS-PAGE gel. Subsequently, the proteins were transferred onto a PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA) at 110 V for 1 hour at 4°C. After the transfer, the membrane was blocked using a 5% blocking buffer, consisting of 5% non-fat dried milk and 0.1% Tween-20 in TBS, for 1 hour at room temperature. The membrane was then incubated with a specific primary antibody overnight. Following the primary antibody incubation, the membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) and subsequently incubated with a secondary antibody at room temperature for 1 hour. After another round of washing with TBS-T, the signal was visualized by exposing the membrane to X-ray film using Clarity Max ECL Western Blotting

substrate (Bio-Rad, USA). ImageJ software was used for relative quantification of the band intensities by comparing to the control samples.

3.9 Lysosomal acid phosphatase assay

Following treatment, the cells were gathered and subjected to lysis using Passive Lysis Buffer, which consists of 25 mM Tris-HCl (pH 7.8), 2 mM DTT, 2 mM EDTA, 10% glycerol, and 1% Triton X-100. This lysis process was carried out for 30 minutes on ice. The protein concentration was determined through a Bradford assay. To assess acid phosphatase activity, ten micrograms of the lysate were incubated with 5 mM pNPP in 100 µl of citrate buffer (pH 4.8, 90 mM) in a 96-well plate at 37°C for 30 minutes. The reaction was halted using a 100 mM NaOH solution, and the absorbance was measured at 405 nm using a microplate reader from Thermo Fisher Scientific, USA.

3.10 Fluorescence microscopy

Cells seeded in 24-well plates were transfected with the mCherry-GFP-LC3 construct and then exposed to the provided chemical treatments 24 hours after transfection. To analyze the cells, fluorescence microscopy was carried out using the Nikon Eclipse Ti2-E inverted fluorescence microscope, and the resulting images were processed with ImageJ software. To ensure consistency, the detectors' gain, offset levels, and laser power were carefully calibrated and remained unchanged throughout the experiment. Both the number and type (yellow or red) of puncta in a minimum of 30 cells were manually counted and then a graph illustrating the average number of LC3 puncta per cell was created.

3.11 Confocal microscopy

To evaluate mitochondrial morphology, cells were first transfected with MT-RFP and then subjected to the specified chemical treatments 24 hours after transfection. Images were captured using a LSM 710 inverted confocal microscope from Carl Zeiss, Germany. The calibration settings

for detectors, including gain, offset levels, and laser power, were set consistently and remained unchanged throughout the experiment.

In a similar manner, cells were treated with the provided chemicals for a duration of 24 hours. Following treatment, they were stained with LysoTracker Blue (Invitrogen) and subsequently imaged using the LSM 710 inverted confocal microscope. As with the mitochondrial imaging, the detectors' gain, offset levels, and laser power settings were maintained at identical levels and remained constant for the entire experiment. The number of lysosomes per cell was quantified using the ITCN plugin within the ImageJ software.

3.12 ATP assay

Cellular ATP levels were quantified using an ATP determination kit from Molecular Probes/Life Technologies in Canada. This involved utilizing a 1:10 dilution of cell lysate mixed with an ATP determination master mix containing 25 mM Tricine buffer (pH 7.8), 5 mM $MgSO_4$, 0.5 mM D-luciferin, 1.25 µg/ml firefly luciferase, 100 µM EDTA, and 1 mM DTT. The luminescence intensity was then measured using the TriStar^2 LB 942 Multimode Microplate Reader by Berthold Technologies in Germany. To account for variations in protein content, the readings were normalized using data obtained from a Bradford assay.

3.13 Mitochondrial complex I assay by spectrophotometric method

The mitochondrial complex I activity was assessed through spectrophotometric analysis. After the designated treatment, the cells underwent 2-3 freeze-thaw cycles in a solution containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), 40 mM KCl, 2 mM EDTA, supplemented with 1 mg/ml of fatty acid-free BSA, 0.01% digitonin, and 10% Percoll. Following this, the cells were washed three times with a freeze-thaw solution lacking digitonin and then resuspended in a complex I assay buffer composed of 35 mM potassium phosphate (pH 7.4), 1 mM EDTA, 2.5 mM NaN_3, 1 mg/ml BSA, 2 µg/ml antimycin A, and 5 mM NADH.

The enzymatic reaction was initiated by adding 80 µg of cell lysate to 500 µl of the assay buffer in a 1-ml quartz cuvette. Complex I activity was monitored for 2 minutes by measuring the

decrease in absorbance at 340 nm after the addition of 2.5 mM acceptor decylubiquinone, which indicated the oxidation of NADH.

3.14 Exosome treatment

Exosomes were isolated and exosomal protein was quantified by Bradford's Assay and 60 µg/ml of exosomes were incubated with cells at a density of 5×10^4 cells/per well in 24 well plate, or 3×10^4 cells/per well in 48 well plate. Exosomes were resuspended in exo-depleted media and cells were incubated with the exosomes for different time periods and analyzed.

The serum-derived exosomes were isolated using the method described above, and exosomal protein was quantified using BCA (Bicinchoninic) assay. 50µg/mL of exosomes were incubated with primary cortical and midbrain neurons at a density of 75,000 and 100,000 cells/per well in 48 well plates. Exosomes were resuspended in sterile PBS and primary neurons were incubated with the exosomes for a period of 24h.

3.15 miRNA loading into exosomes

Both conditioned media (CM) and a miRNA mimic were introduced into the cells using the Lipofectamine RNAiMAX transfection reagent from ThermoFisher Scientific, Invitrogen, USA. After 12 hours post-transfection, the media was replaced with pre-conditioned media. Subsequently, 24 hours after the media change, the pre-conditioned media was harvested, and the exosomes were isolated using the protocol described earlier.

In a similar manner, neuronal cells that had been pre-exposed to PD stress inducers, 6-OHDA and Rotenone for 24 hours, were then treated with exosomes loaded with miRNA mimic for the specified durations. These miRNA mimic-loaded exosomes, with a concentration of 60 µg/ml, were resuspended in media depleted of exosomes and incubated with the cells.

3.16 miRNA expression analysis by Next Generation Sequencing (NGS)

To investigate the selective enrichment of exosomal miRNAs in SK-N-SH neuroblastoma cells under both untreated and 6-OHDA treated conditions, Next Generation Sequencing (NGS) was employed. Small RNAs were extracted using the miRCURY™ RNA Isolation Kit (Exiqon). Subsequently, library preparation was carried out using the NEBNext® Multiplex Small RNA Library Prep kit. These prepared libraries were then sequenced on an Illumina HiSeq2500.

The reads obtained were pre-processed and aligned to the reference human genome (hg19 version) and miRBase version 21. Differential expression analysis was performed using DESeq2. The interactions between miRNAs and mRNA targets were explored using the ENCORI/starBase database (http://starbase.sysu.edu.cn/) [269]. Target pathways for the selected miRNAs were determined by combining all miRNAs of each category, the amalgamation of all five target prediction tools, and ClipSeq with low stringency, while ensuring a corrected p value of less than 0.05.

Gene Ontology (GO) terms and pathways were retrieved for each category [270]. To visualize the cellular targets of the two selected miRNAs, Cytoscape software was employed.

3.17 RNA isolation and RT-qPCR

Extraction of total RNA from both cells and exosome samples was done using TRIzol reagent from Takara, Japan. To prepare small RNA for analysis, a poly-A tail was added using *E. coli* Poly-A Polymerase from New England Biolabs, UK, incubating at 37°C for 30 minutes, followed by enzyme inactivation at 65°C for 5 minutes. Subsequently, cDNA synthesis was conducted with a kit from Takara, Japan. The primers for miRNAs were designed based on previous reports.

The levels of miRNAs were determined using the $2^{-\Delta\Delta CT}$ method, with 5S rRNA, U6 snRNA, or U87 serving as endogenous controls for miRNA analysis. The PCR conditions involved an initial step at 95°C for 2 minutes, followed by 35 cycles of 95°C for 5 seconds and 60°C for 1 minute (for miRNA). Melt curves were obtained to ensure the specificity of the reactions.

3.18 Droplet Digital PCR

EvaGreen chemistry was employed for all the ddPCR assays conducted in the study. To standardize the amplification of both miRNAs, a variety of primer concentrations (with a consistent 10nM concentration) and annealing temperatures were tested. In each experiment, 1ng of cDNA was used. In brief, an amplification mixture of 20 µl, along with 70 µl of droplet generation oil specifically designed for EvaGreen (Bio-Rad), was loaded into a disposable droplet generator cartridge (Bio-Rad). Using a QX200 droplet generator (Bio-Rad), droplets were created and then transferred to a 96-well PCR plate (Bio-Rad). This plate was then placed in a standard thermal cycler (Bio-Rad). The PCR cycling conditions were as follows: an initial denaturation at 95℃ for 5 minutes, followed by 40 cycles of denaturation at 95℃ for 30 seconds and annealing at 58.5℃ for 1 minute. Three final steps included incubation at 4℃ for 5 minutes, 90℃ for 5 minutes, and a final hold at 4℃ for 30 minutes. After the PCR cycles were completed, the plate was analyzed using the QX200™ Droplet Reader and further processed using the QuantaSoft™ software. The results are presented in terms of copies/µl. Two types of droplets were distinguished: (1) positive droplets (shown in blue), indicating the amplification of the respective miRNAs, and (2) negative droplets (in grey), indicating the absence of the respective miRNAs.

3.19 Cell death assay by MTT

Cell viability was assessed through the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. The MTT assay was executed by incubating the cells with MTT (0.1 mg/ml) provided by ThermoFisher, at 37℃ for 1–2 hours. Subsequently, the visible violet crystals generated by the MTT reaction were dissolved in DMSO, and the absorbance was measured at 570 nm using a microplate reader.

3.20 Cell death detection by ELISA

Cell death detection by ELISA was carried out using a kit-based assay from Roche, Germany. Following treatment, the cells were harvested and lysed using the lysis buffer provided in the kit, and the lysate was subsequently centrifuged at 8000g for 10 minutes to remove cellular debris.

The resulting supernatant, containing cytoplasmic proteins, was incubated on streptavidin-coated 96-well plates along with biotin-tagged anti-histone and anti-DNA-POD antibodies.

After a 2-3 hour incubation period, the mixture was removed from the plates, and the plates were washed three times. Subsequently, the substrate solution from the kit was added, resulting in the development of a blue-purple colored solution. The enzymatic reaction was halted using a stop buffer, and colorimetric readings were taken for the samples at 405nm and 490nm. The enrichment factor was calculated using the formula: mU sample/mU control.

3.21 Caspase 3/7 activity

Caspase 3/7 activity was measured for the cells post-treatment by using Caspase-Glo® 3/7Assay Systems (Promega) as per the manufacturer's protocol.

3.22 ROS estimation and mitochondrial membrane potential

To assess the levels of total cellular reactive oxygen species (ROS) and mitochondrial ROS, staining with CM-H2DCFDA (10 μM) and MitoSox™ Red (5 μM) was employed, respectively.

After the treatment, the cells were stained with MitoSox™ and examined under a fluorescence microscope (Nikon Ti2-E inverted fluorescence microscope, Japan). A minimum of 10 images and 80-100 cells for each condition were analyzed. Additionally, quantification of ROS levels was obtained using fluorometry. In this procedure, cells were treated and stained with CM-H2DCFDA (10μM) in DPBS to quantify intracellular ROS. Following staining, the cells were washed with DPBS and normalized using a Bradford assay. Fluorescence intensity was determined with a fluorometer from Hitachi High-Technologies Corp., Japan, with excitation/emission wavelengths set at 495/520–540 nm.

The mitochondrial membrane potential was assessed by staining the cells with Tetramethylrhodamine (TMRM) at a concentration of 5μM for 15 minutes, followed by quantification of fluorescence at 510/570–600 using a fluorimeter. To ensure consistency, determination of protein content was done with the Bradford assay and used for assay normalization.

3.23 Immunocytochemistry

To assess specific dopaminergic neurotoxicity in primary midbrain neurons, immunocytochemistry was employed. Following the treatment, the cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at 4ºC and then rinsed with PBS. After rinsing, the cells were subjected to an incubation with primary antibodies, namely Tyrosine hydroxylase (TH) (AB9983) from Millipore, Sigma, USA, which is specific to dopaminergic neurons, and MAP2 (AB5622) from Millipore, Sigma, USA, serving as a general neuronal marker. This incubation took place at 4ºC overnight. The primary antibody was prepared in 1X PBS containing 10% normal donkey serum (NDS) and 0.3% Triton-X 100. After the overnight incubation with the primary antibody, the cells were washed with PBS three times for 5 minutes each. Subsequently, the cells were incubated with the corresponding fluorescent-tagged secondary antibody for 2 hours at room temperature. After another three washes with PBS, the cells were imaged using a confocal microscope (Nikon) with a 20X objective.

3.24 Details for primer sequences

Sr. No.	Gene name	Primer type	Primer sequence (5'-3')
1	hsa-miR-664b-5p	miR Seq	UGGGCUAAGGGAGAUGAUUGGGUA
		F	GGAGTGGGCTAAGGGAGATGA
		R	CGCAGGTTTTTTTTTTTTTTTACCCAA
2	hsa-miR-320d	miR Seq	AAAAGCUGGGUUGAGAGGA
		F	GCGCAGAAAAGCTGGGTTGA
		R	GCAGGTCCAGTTTTTTTTTTTTTTTCCT
3	hsa-miR-92a-1-5p	miR Seq	AGGUUGGGAUCGGUUGCAAUGCU
		F	CGGAGGTTGGGATCGGTTG
		R	CAGGTCCAGTTTTTTTTTTTTTTTAGCATT
4	hsa-miR-181c-5p/ rno-miR-181c-5p	miR Seq	AACAUUCAACCUGUCGGUGAGU
		F	CAGGAACATTCAACCTGTCGGT
		R	CGGGTCCAGTTTTTTTTTTTTTTTACTC

5	hsa-miR-484/ rno-miR-484	miR Seq	UCAGGCUCAGUCCCCUCCCGAU
		F	CCGTGAAGTCAGGCTCAGTC
		R	GCGGTCCAGTTTTTTTTTTTTTTTATCG
6	hsa-miR-30a-5p/ rno-miR-30a-5p	miR Seq	UGUAAACAUCCUCGACUGGAAG
		F	GGCAGTGTAAACATCCTCGAC
		R	CAGGTCCAGTTTTTTTTTTTTTTTCTTCC
7	hsa-miR-122-5p	miR Seq	UGGAGUGUGACAAUGGUGUUUG
		F	CGCAGTGGAGTGTGACAATG
		R	CAGGTCCAGTTTTTTTTTTTTTTTCAAAC
8	hsa-miR-98-5p	miR Seq	UGAGGUAGUAAGUUGUAUUGUU
		F	GCGCAGTGAGGTAGTAAGTTGT
		R	GCAGTTTTTTTTTTTTTTTTAACAAT
9	hsa-let-7a-5p	miR Seq	UGAGGUAGUAGGUUGUAUAGUU
		F	GGCAGTGAGGTAGTAGTTTGT
		R	GGTCCAGTTTTTTTTTTTTTTTAACTGTAC
10	hsa-miR-5701	miR Seq	UUAUUGUCACGUUCUGAUU
		F	GCAGGCAGTTATTGTCACGT
		R	GTCCAGTTTTTTTTTTTTTTTTAATCAG
11	hsa-miR-320a	miR Seq	AAAAGCUGGGUUGAGAGGGCGA
		F	CAGAAAAGCTGGGTTGAGAG
		R	GTTTTTTTTTTTTTTTCGCCC
12	rno-miR-320a-3p	miR Seq	AAAAGCUGGGUUGAGAGGGCGA
		F	CGCAGAAAAGCTGGGTTGAGA
		R	TGCAGTTTTTTTTTTTTTTTCGCCC
13	rno-miR-93-5p	miR Seq	CAAAGUGCUGUUCGUGCAGGUAG
		F	GCAGGCAAAGTGCTGTTCGTG
		R	GCGTCCAGTTTTTTTTTTTTTTTCTACCT
14	rno-miR-708-5p	miR Seq	AAGGAGCUUACAAUCUAGCUGGG
		F	CGCAGAAGGAGCTTACAATCTAGC
		R	GGGTCCAGTTTTTTTTTTTTTTTCCCA

Endogenous controls			
1	5S rRNA (human)	F	GGTCTACGGCCATACCACC
		R	CAGTTTTTTTTTTTTTTAAAGCCTACAG
2	U6 snRNA (human)	F	GGTGCTCGCTTCGGCA
		R	TCCAGTTTTTTTTTTTTTTAAAAATATGGAAC
3	U87 (small nucleolar RNA U87) (rat)	F	GCAGACAATGATGACTTATGTTTTTGC
		R	GGTCCAGTTTTTTTTTTTTTTTGCTCAGT

Table 3.1. Details of primer sequences.

3.25 Statistical analysis

Data are shown as mean ± SEM for number of times the experiment was repeated. Comparisons for columns were performed using student t-test for repeated measurements to determine the levels of significance for each group. Comparisons between groups was done by either one-way or two-way ANOVA, using Dunnett's (comparison back to control), Tukey's (comparison of all groups) or Sidak's (where only select comparisons were scientifically warranted) multiple comparisons test. The experiments were performed a minimum of three times independently and $p<0.05$ was considered as statistically significant. GraphPad Prism (version 8 and 9) software was used to perform all the statistical analysis.