Chapter II

Materials and methods

2.1 Cell culture

All the plastic wares for the cell culture (Eppendorf or Tarson), Dulbecco's Modified Eagle Medium (DMEM) (Gibco), DMEM/F12 (Gibco), FBS serum (Biosera), HBSS (Gibco), Poly-l-lysine (Sigma), DNase I (Himedia), Trypsin Phosphate Versene Glucose (TPVG) (Himedia), antibiotic antimycotic solution (Himedia) were used in vertical laminar hood and dissecting surgicals were autoclaved to maintain the sterility. Use, care and housing of all animals were done in compliance with Institutional Animal Ethical Committee (IAEC), Departments of Biochemistry and Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda.

2.1.1 Primary astrocytes isolation

Cortical astrocytes were isolated from Charles foster neonatal rat pups (post natal day P0-2)by a well described method (Chen et al. 2007, Sharma et al. 2015). In brief, in the sterile conditions cerebral cortex was isolated by removing cerebellum, olfactory bulbs, basal ganglia and hippocampus followed by the removal of meninges in ice cold 1X HBSS. These meninges-free cortices were diced, and digested at 37 $^{\circ}$C with 0.2mg/ml DNase I and 0.25% TPVG in 1X HBSS. After attaining single cell suspension following centrifugation and passing through 70µm nylon cell strainer, cells were plated in poly-l-lysine (PLL-10µg/ml) coated flasks with complete medium-DMEM containing high glucose, 4mM L-Glutamine supplemented with 10% FBS and 1% penicillin and streptomycin;and then flasks were incubated at 37 $^{\circ}$C with 5% CO_2. Cells were feeded with complete medium change every 2-3 days until 10th day *in vitro* (DIV). At 10th DIV, mixed glial cultures were confluent, process-bearing OPCs appeared on top of phase-gray bed layers of astrocytes. Microglial cells were removed by shaking flask horizontally at 200 r.p.m. for 1 hrfollowed by OPCs for overnight at 37°C.After removing the cell suspension post-shaking, adherent pure astrocytes (98-100%) grown in monolayer were obtained.These astrocytes were also grown on complete medium, counted using the Trypan blue exclusion assay with a hemocytometerand were sub-cultured on the day before the experiment in PLL coated 6, 12, 24,96 well plates or coverslips for immunocytochemistry.

2.1.2 C6 cell line culture

C6 glial cell line (Benda et al. 1968) was acquired from National Centre for Cell Science (NCCS-Pune, India) and maintained at 5% CO_2 and 37°C in DMEM/F12 medium (Gibco) supplemented with 10% FBS and 1% Penicillin and Streptomycin (Singh, Sharma, and Pillai 2017, Sharma et al. 2018).

2.2 Cell treatments

2.2.1 Quercetin hydrate treatment

Quercetin hydrate (Sigma, #337951) is immiscible in cell culture medium. However, 1mM quercetin hydrate stock solution was prepared in 10% DMSO freshly. The cells were incubated with different quercetin hydrate concentrations (25, 50, 100 and 200 μM) in DMEM (for astrocytes) and DMEM/F12 (for C6 cells) containing 1% antibiotic.

2.2.2 DHA treatment

Docosahexaenoic acid (Sigma, #D2534) is in oil form and immiscible in medium. So 10 mM stock was prepared freshly in cell culture medium supplemented with (1.5mg/ml) bovine serum albumin(BSA) (DHA: BSA ratio was 2:1) (Zhao et al. 2005). The cells were incubated with different DHA concentrations (25, 50, 100 and 200 μM) in DMEM (for astrocytes) and DMEM/F12 (for C6 cells) containing 1% antibiotic.

2.2.3 MeCP2 siRNA transfection

siRNAs were dissolved in nuclease free water and 10 μM stocks were stored at -20°C. After the pre-incubation with QH or DHA, the cells were transfected with 10-20 nM universal negative siRNA or MeCP2 siRNA (SASI_Rn01_00072926, Sigma) for 24 hrs using hiperfect reagent (Qiagen) as per the manufacturer instructions (Sharma et al. 2015).

2.2.4 LPS treatment

10mg/ml lipopolysaccharide (E.coli O127:B8, Sigma, Cat. No. L3129) stock was prepared in DMEM medium and stored at -20°C. Cells were treated with 1-5μg/ml LPS in DMEM containing 1% antibiotic as per the experiments described in later chapters.

2.3 Cell viability

2.3.2 MTT assay

For the assessment of effects of treated molecules on cell viability, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, astrocytes/ C6 cells were seeded in 96 well plates and after 24 hrs treatments; the medium was replaced with 100µl fresh media containing 0.1mg/ml MTT. After 3 hrs, MTT was removed and the crystals of formazan were dissolved in DMSO. Formazan was quantified at 570 nm using a microplate reader ELx800-MS (Bio Tek) (Singh, Sharma, and Pillai 2017).

2.4 Immunocytochemistry

For the characterization and protein expression analysis, astrocytes/ C6 cells grown on PLL-coated coverslips were fixed in 4% paraformaldehyde (15 min) in 1X PBS (pH 7.4) at room temperature. Cells were washed twice (5 min each) with ice cold PBS and permeabilized by incubating with 0.25% tritonX-100 (10min) followed by 3 washes (5 min each) with 1X PBS and then blocking (30 min) with 1% BSA in PBS-T (0.1% tween 20). Primary antibodies (Table 2.1) diluted in blocking buffer were incubated for overnight at 4°C in humified chamber. Cells were washed thrice (5 min each) to remove excess primary antibody. In dark, cells were incubated with respective fluorophore labeled secondary antibodies (Table 2.1) for 1 hr at room temperature followed by nucleus staining with DAPI (0.1 µg/ml). Cover-slips were mounted with 80% glycerol and sealed with nail polish. Slides were imaged under either confocal (Carl Zeiss) or Floid (Invitrogen) microscopes at 40X/60X (Singh, Sharma, and Pillai 2017).

Table 2.1 Antibodies used for Immunocytochemistry

Primary antibody	Dilution	Secondary antibody	Dilution
GFAP	1:100	Goat-FITC	1:100
Iba-1	1:50	Mouse-FITC, Mouse-TRITC	1:50

2.5 Intracellular Ca^{2+} measurement by Fluorometry

Following the 24 hrs MeCP2 siRNA incubation, the cells were incubated with the calcium sensitive fluorescent dye, Cal 520AM (4µM) (Abcam, # ab171868) dissolved in DMEM (with 1% antibiotic) in dark for 2 hrs in incubator (at 37°C, 5% CO_2 and 95% O_2), then cells were washed

with and put in 1X HBSS (without Ca^{+2} and Mg^{+2}) followed by $[Ca^{2+}]_i$ measurement by the fluorescence micro plate reader (Synergy HT) at Excitation: Emission: 485/20: 528/20 wavelengths and gain 35% within 30 mins.

2.6 H2.DCF.DA staining and fluorometric analysis

ROS generation was measured using H2.DCF.DA dye as previously described (Wang and Joseph 1999). In brief, before MeCP2 siRNA transfection, cells were loaded with 100µM H2.DCF-DA (Sigma, # D6883) in DMEM/F12 in dark for 30 mins in incubator (at 37°C, 5% CO2 and 95% O2). Further the H2.DCF-DA was removed and cells were washed with 1X PBS followed by 24 hrss MeCP2 siRNA incubation as discussed earlier. Fluorescence was measured by the fluorescence micro plate reader (Synergy HT) at Excitation: Emission: 485/20: 528/20 wavelengths and gain 35% within 30 mins.

2.7 Semi quantitative and quantitative real-time RT-PCR

Total RNA was harvested by trizol reagent (Invitrogen) and integrity-purity was checked by running RNA on 1% agarose gel in sterile condition. RNA was quantified by Qubit RNA assay kit in Qubit 2.0 fluorometer (Invitrogen) and 1µg of total RNA was used to prepare cDNA using cDNA kit (Verso-Thermo Fisher, #AB1453). A total 1µl cDNA was amplified by PCR and the PCR products were run on 2% agarose gel and visualised in gel doc (Bio-rad). Band intensity was measured by Image J software (1.51j8) and normalized to GAPDH. Quantitative real-time PCR was performed in a QuantStudio 12K (Life Technology) real-time PCR instrument using SYBR-green PCR master mix (Applied Biosystems) and the data were analyzed by $2^{-\Delta\Delta CT}$ (Livak) Method (Livak and Schmittgen 2001). Primers used for this analysis are as in table 2.2.

Table2.2 Species-specific oligonucleotide primers list

Target gene	Primer sequence (5'→3')	Annealing temperature (°C)	Amplicon (bp)	Reference
Ndufv2	FP: GCCAGTTGGGAAGTACCACA	60	224	Present study
	RP:CCTTGGGTGTCAGATCCTCG			
Uqcrc1	FP:TTGACGTTGGCAGTCGCTAT	58	290	
	RP:CTCCCGCAGGATCACATCTC			
MeCP2	FP:CATGGTAGCTGGGATTTAG	60	203	
	RP:GAGCTTTCTGATGTCCTGC			
GFAP	FP: GCCTCTCCCTGTCTCGAATG	60	182	Present study
	RP:CGCCTTGTTTTGCTGTTCCA			
BDNF	FP: CCATAAGGACGCGGACTTGT	60	65	(Fuchikami et al. , 2009)
	RP: GAGGCTCCAAAGGCACTTGA			
IL-1β	FP: TGTCTGAAGCAGCTATGGCAA	60	228	(Yabe et al. , 2005)
	RP: TCTCCACAGCCACAATGAGTGA			
IL-6	FP: AGCCAGAGTCATTCAGAGCAATACTG	60	225	
	RP: CACTAGGTTTGCCGAGTAGACCTC			
TNF-α	FP: CCAGAACTCCAGGCGGTGTCTGTG	60	226	
	RP: GTGGTTTGCTACGACGTGGGCTAC			
COX-2	FP: CCATGTCAAAACCGTGGTGAATG	58	374	(Yin et al. , 2002)
	RP: ATGGGAGTTGGGCAGTCATCAG			
HO-1	FP: CTTTCAGAAGGGTCAGGTGTC	58	102	(Lin et al. , 2011)
	RP: TGCTTGTTTCGCTCTATCTCC			
TLR-4	FP: AGT TGG CTC TGC CAA GTC TCA GAT	60	144	(Gárate et al. , 2011)
	RP: TGG CAC TCA TCA GGA TGA CAC CAT			
GAPDH	FP: AGACAGCCGCATCTTCTTGT	60	200	(Swiss et al. , 2011)
	RP: CTTGCCGTGGGTAGAGTCAT			

2.8 Western blotting

For analyses of protein expression, 2-5×10^5 cells were exposed to various treatments. Post treatments, the cells were washed twice with sterile 1X PBS (Himedia) and cell lysates were prepared in 200µl 2X Laemmli sample buffer**.** Lysates were stored in -20°C deep freezer and total protein was quantified by Qubit protein assay kit in Qubit 2.0 fluorometer (Invitrogen) on the day of western blotting as per the manufacturer's instruction. 40-50 µg of total protein in 2X Laemmli sample buffer (with 0.2% bromophenol blue, (BPB)) was denatured by heating in boiling water bath for 5 mins and resolved by 10% SDS-polyacrylamide gel electrophoresis (100V) until the blue dye front reaches the bottom. Protein was wet transferred on 45µm nitrocellulose membrane with constant voltage (100V) for 90 min in cooling condition (Biorad unit) to prevent gel and transfer buffer from overheating. To avoid non-specific binding of antibodies, membrane was incubated with blocking buffer i.e. 5% non-fat milk for non-phosphorylated and 3% BSA for phosphorylated forms for 1 hr with agitation. The membranes were probed with primary antibodies (Table 2.3) prepared in blocking buffer for overnight at 4°C followed by 3 washes in 1X TBST (0.5% tween20) for 5 mins with agitation with orbital shaker. Each membrane was incubated with HRP-conjugated secondary antibodies (Table 2.3) at room temperature for 1 hr followed by 3 washes in 1X TBST as above. Bands were visualized using ECL reagent (Invitrogen) and developed on X-ray films or chemi-doc (Fusion SL with Vision Capt v16.12 software). The intensity of bands were measured by Image J software (1.51j8) and were normalized to intensity of internal control β-actin.

Table 2.3Antibodies used in western blot analysis

Primary antibody	Dilution	Secondary antibody (HRP conjugated)	Dilution	Molecular Weight (kDa)
pERK1/2 R&Dsystems#af1018	1:800	Rabbit Sigma#A0545	1:2500	42, 44
Phospho-p38 MAPK pThr180/Tyr182 Pierce#MA5-15218	1:1000	Mouse Sigma#A9044	1:5000	43
GFAP Pierce# PA3-16727	1: 5000	Rabbit	1:10000	55
GFAP Abcam,# ab53554	1:1000	Goat Sigma#A5420	1:2500	50
p-IκB-α Santa Cruz,#sc-8404	1:1000	Mouse	1:5000	41
β-Actin-HRP Santa Cruz,#sc-47778	1:1000	--		41
Ndufv2 Santa Cruz, # sc-68887	1:200	Rabbit	1:3000	24
MeCP2 Santa Cruz, #sc20700	1:200	Rabbit	1:3000	55,75
Iba-1 Santa Cruz,#sc-32725	1:200	Mouse	1:3000	17

2.9 Mitochondrial enzyme activity assays

For mitochondrial ETC enzyme activity assays, cell pellets were suspended in potassium phosphate buffer (0.5M, pH 7.5) and lysed by take up and expelling the suspension for several (~ 40) times using 23 gauge needle syringe until homogenous solution appeared. Total protein concentration was measured by Bradford method (Sigma) and 20µg protein was used for each assay as recommended by Spinazzi et al. (2012). The enzymatic activities were performed and calculated as nmol min^{-1} mg^{-1} of protein according to the previously described (Spinazzi et al. 2012) method with minor modification of KCN replaced with 100 mM sodium-azide which has an action on respiratory chain very similar to cyanide (Bowler et al. 2006, Chen and Lesnefsky 2006). The specific activity of complexes were calculated by subtracting inhibitor-resistant activity (with inhibitor) from total activity (without inhibitor) (Inhibitors- C-I: rotenone, C-II: malonate, C-III: antimycin, C-IV: Na-azide).

2.10 Mitochondrial membrane potential (MMP) measurement

Followed by 24 hrs MeCP2 siRNA treatment, 0.5 μM rhodamine 123 (cationic dye) (sigma, # R8004) dissolved in DMEM (with 1% antibiotic) was incubated in dark for 30 mins in incubator (at 37°C, 5% CO2 and 95% O2), then cells were washed with and put in 1X HBSS (without Ca^{+2} and Mg^{+2}) followed by MMP measurement by the fluorescence micro plate reader (Synergy HT) at Excitation: Emission: 485/20: 528/20 wavelengths and gain 35% within 30 mins (Shulyakova 2016).