CHAPTER 2

MATERIALS AND METHODS

2.1 Mixed glial cell isolation

Mixed glial cultures were isolated from cerebral cortices of P0-P2 day rat pups (Chen et al., 2007; Frost et al., 2009). The cerebra were dissected, diced into smaller pieces and subjected to enzymatic digestion with DNase I (stock solution- 0.2mg/ml) and trypsin (stock solution- 0.25%) in HBSS at 37^0C, to generate a single-cell suspension. Cells were plated onto PLL coated T-75 cm^2 tissue culture flasks in DMEM containing 10% FBS and 1% penicillin/streptomycin and incubated in a CO_2 incubator at 37^0C for 10 days with a media change on every third day. After 6-8 days, mixed glial cultures are observed with microglia and OPCs growing on an underlying astrocytic bed. After 10-12 days, flasks were sealed and shaken on an orbital shaker at 150rpm for 1hr at 37^0C to remove microglia. Cultures were refed and shaken again at 200rpm for 18-20 hrs at 37^0C followed by differential adhesion for 1hr on non-tissue culture petri dish to separate OPCs from an underlying monolayer of astrocytes. The cell suspension was passed through 40μm pore size filter and the collected suspension was centrifuged at 100g for 10mins. The cell pellet was dissociated in DMEM medium and cultured in Sato medium (Bottenstein and Sato, 1979). PDGF-AA was added at 10ng/ml to Sato medium for proliferation of OPCs (except in the proliferation assay).

Underlying cultures were enriched with >95% GFAP-positive astrocytes as confirmed by immunocytochemical analysis. Astrocytes were dissociated with 0.1% trypsin and passaged using DFG medium for further experiments. Animal protocols were duly approved by Institutional Animal Ethical Committee (IAEC), Department of Zoology and Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda. The protocol numbers are mentioned herewith: ZD/13/2014, ZD/34/2014, ZD/03/2016, MSU-Z/IAEC/07-2017

2.2 Dorsal Root Ganglion neuron (DRGN) cell isolation

Dorsal Root Ganglion (DRG) cultures were generated from P7-P9 day old rat pups (O'Meara et al., 2011). The spine was extracted and transferred to a clean Petri dish. Surrounding muscles and bone were trimmed off from the spine, this eases the dissection of DRGs. The trimmed spine was transferred to a new Petri dish with the ventral side up and cut medially through the spinal column in a longitudinal fashion. The spinal column was gently opened to expose the spinal cord. DRGs are found beneath and lateral to the spinal cord and were gently isolated using fine tipped forceps while avoiding damage to the ganglia. Extracted DRGs were transferred to antibiotic-free ice cold HBSS in a new Petri dish. The excessively long roots attached to the DRG were cut to avoid any contaminating cells and fibroblasts. The trimmed DRGs were then re-suspended in HBSS, centrifuged and subjected to enzymatic digestion by Papain (5mg/ml) followed by Collagenase A (4mg/ml) solution for 20-30 minutes each at 37^0C. The cell suspension was thoroughly triturated and suspended in DMEM containing FBS and incubated in a petri dish for 1.25 hours at 37^0C to remove any contaminating cells which will have adhered to the petri dish. DRGNs, observed as large black bodied cells under the microscope were collected by centrifugation, re-suspended in appropriate volume of media and plated on Fibronectin (10µg/ml) coated culture vessels. Cells were grown in DMEM containing 10% FBS at 37^0C and 5% CO_2 with a media change every alternate day for 9-10 days to obtain an extensive neurite bed.

2.3 Establishment of Myelinating Co-Culture: [Astrocytes-DRGN-Oligodendrocytes]

For astrocyte-DRGN-oligodendrocyte co-cultures, astrocytic monolayer was first transfected twice with siRNA duplexes specific for rat MeCP2 or with a universal negative control siRNA using HiPerfect transfection reagent (Qiagen) according to the manufacturer's protocol. DRGN culture was plated onto the astrocytes and grown for 3 days during which the DRGN form a good dendrite network. On the 3rd day, OPCs were gently plated onto the astrocyte-DRGN cells without disrupting them. The triple culture was grown for another 3 days to allow the interaction between

oligodendrocytes and DRGN thus facilitating myelination to take place. Cultures were maintained at 37^0C incubator at 5% CO2, after which cell lysates were collected for further analysis.

For oligodendrocyte-DRGN co-cultures, a complete media change was performed on DRGNs. OPCs were gently added from the OPC enriched cell suspension onto the DRGN monolayer in 12 well plate without disrupting it. The cultures were incubated in 37^0C incubator with minimal movement of the culture plate until all cells have attached.

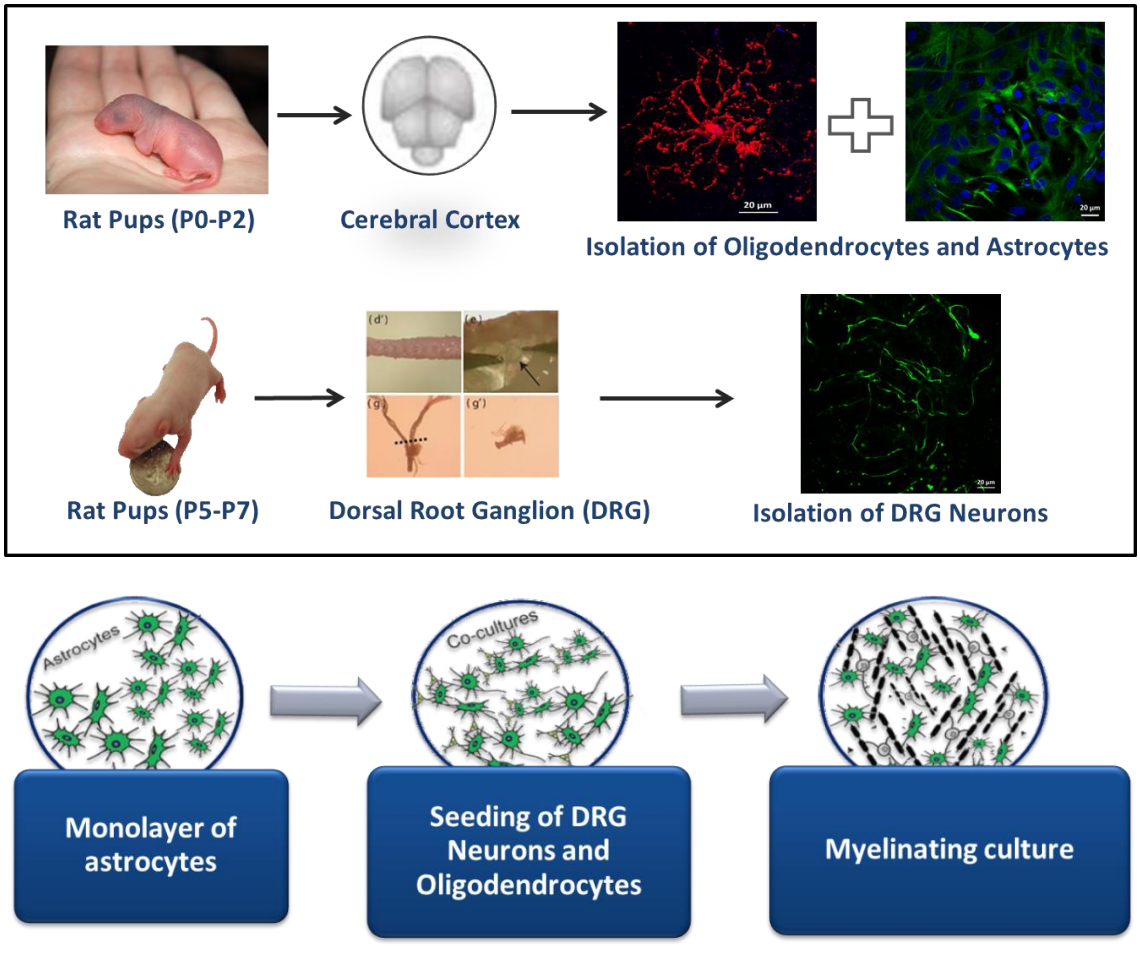

Figure 2.1: Schematic diagram of establishment of myelinating co-culture (Astrocytes-DRGN-Oligodendrocytes).

2.4 siRNA transfection

Cells were transfected with siRNA duplexes (predesigned and synthesized by Sigma) specific for rat MeCP2 (siRNA ID: SASI_Rn01_00072926) or with a Universal negative control siRNA, using HiPerFect transfection reagent (Qiagen) according to the manufacturer's protocol. The transfection was repeated after 24 hrs of first transfection for optimal silencing. RNA, proteins and condition media were isolated after 24 hrs of second transfection.

2.5 Real time PCR

Total RNA was isolated from cells using Trizol reagent (Sigma) following the manufacturer's protocol. RNA was quantified using a Qubit RNA assay kit (Invitrogen) in Qubit 2.0 Fluorometer (Invitrogen). Total RNA (1µg) was used for a 10µl reverse transcription (RT) reaction using verso cDNA synthesis kit (Thermo Scientific). Quantitative RT-PCR was performed using SYBR Select Master Mix (Applied Biosystems) in QuantStudio12K (Life Technology) real-time PCR machine with primers to detect selected messenger RNA (mRNA) targets. Specificity of the products was confirmed by measuring the melting curve of each sample. Data were normalized to the internal control GAPDH and analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primers used for qPCR are listed in Table 1.

Table 1: List of Primers

Primers	Sequence	References
Gapdh	Forward: AGACAGCCGCATCTTCTTGT Reverse: CTTGCCGTGGGTAGAGTCAT	(Swiss et al., 2011)
Mecp2	Forward: GACCGGGGACCTATGTATGA Reverse: CAATCAATTCTACTTTAGAGCGA	Current study
Caspr	Forward: GCTCCTTCTGTTGGATGTCT Reverse: GCCACTCTGCTTTTATTGAACC	Current study
Nrg1	Forward: AGCAGACACCAGCTTCAGAC Reverse: CAAGAAGGCAGGGGACCAAA	Current study
Notch	Forward: CAATGGCACAGGGGCTATGA Reverse: TTAGCGGGTTGTACTGGCTG	(Wang et al., 2017)

Nf155	Forward: ACTGGGAAAGCAGATGGTGG Reverse: TCATTTGGAGGTGTTGGGGA	Current study
Cntf	Forward: AGAACCTCCAGGCTTACCGT Reverse: GGCAAAGGCAGAAACTTGGA	Current study
Lif	Forward: TGCCGTCTGTGCAACAAGTA Reverse: GGACCACCGCACTAATGACT	Current study
Pdgfa	Forward: AGGACGCGTAGAACAATCGG Reverse: TTGTCCGCTCTGGTGAAACT	Current study
Cxcl10	Forward: TGGTACTCCCACTACAGCGT Reverse: GAACAGAGCTAGGAGAGCCG	Current study
Mag	Forward: TGTGTAGCTGAGAAGGAGTATGG Reverse: ACAGTGCGATTCCAGAAGGATTAT	(Ghiani et al., 2007)
Mog	Forward: GAGGGACAGAAGAACCCACA Reverse: CAGTTCTCGACCCTTGCTTC	(Swiss et al., 2011)
Mbp	Forward: CTCTGGCAAGGACTCACACAC Reverse: TCTGCTGAGGGACAGGCCTCTC	(Paintlia et al., 2004)
Plp	Forward: GTGTTCTCCCATGGAATGCT Reverse: TGAAGGTGAGCAGGGAAACT	(Ueno et al., 2012)
Gfap	Forward: AGGCCCTGACATCCCAGGA Reverse: CTGCCTCAGGGTGCCGAG	Current study
Bdnf	Forward: CCATAAGGACGCGGACTTGT Reverse: GAGGCTCCAAAGGCACTTGA	(Fuchikami et al., 2009)
Ngf	Forward: TGCATAGCGTAATGTCCATGTTG Reverse: CTGTGTCAAGGGAATGCTGAA	(Aiga et al., 2006)

2.6 Protein Extraction and Quantification, SDS-PAGE and Western Blotting:

Protein Extraction: Whole cell lysate was prepared in 2X lysis buffer containing protease inhibitor. The cell lysates were stored at -20^{0}C till further use.

Protein Quantification: The total protein was quantified using Qubit protein assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen) according to the manufacturer's instructions.

Sample preparation: 5x loading dye was added to 40µg of aliquoted protein of each sample. The solutions were mixed and heated in a boiling water bath for 10 mins and subjected to a pulse spin. Samples were stored on ice till loading onto the gel.

SDS-PAGE: Gel casting: Resolving gel was poured between PAGE plates in the casting assembly, immediately followed by distil water. The gel was left undisturbed and allowed to fully polymerise before the excess water was removed and stacking gel was poured on top of it. The comb was inserted immediately. Cell lysates of equal protein loads (40µg) was resolved by SDS-PAGE (10%) and separated by electrophoresis using a constant voltage of 100V.

Western Blot: After SDS-PAGE, the gel was equilibrated in 1X transfer buffer for 5 mins. From anode to cathode, a transfer stack was prepared as follows: Sponge pad, filter pad, gel, 0.2µM nitrocellulose membrane, filter pad and sponge pad. Transfer was carried out in transfer unit at 100 V for 90 min. Protein transferred on nitrocellulose membrane were visualised by staining the membrane with Ponceau stain to check the quality of transfer. The reversible stain was washed off with water and the membrane processed for antibody probing.

Antibody probing: Transferred membrane was blocked in blocking buffer (3% BSA in TBS-T) for 1hr at room temperature followed by incubation in primary antibodies with constant agitation at 4^0C overnight. Concentrations of primary antibodies are listed in Table 2. After primary antibody incubation, the membrane was washed in washing buffer for 10mins thrice, followed by incubation in corresponding horseradish peroxidase (HRP) conjugated secondary antibodies for 1hr at room temperature. Membrane was again washed in washing buffer thrice for 10mins. Specific immune-reactivity was visualized using ECL kit. Protein bands were imaged manually on X-ray film or observed digitally in chemidoc. Blots were scanned digitally and quantitatively analysed using Image J software.

Table 2: List of antibodies used for western blot		
Antibody	**Dilution**	**Manufacturer**
Anti-MeCP2	1:750	Millipore
Anti-BDNF	1:700	Abcam
Anti-NGF	1:750	Abcam
Anti-GFAP	1:1000	Santacruz
Anti-Tuj1	1:1000	Santacruz
Anti-MBP	1:700	Santacruz
Anti-PLP	1:1000	Abcam
Anti-β-actin	1:5000	Thermo Pierce

2.7 Immunocytochemistry (ICC)

Cells were grown on PLL coated glass coverslips, fixed in cold 4% paraformaldehyde (PFA) (w/v) for 10 mins at room temperature and washed thrice with cold PBS for 10mins each. They were then permeabilized in PBS containing 0.25% Triton X-100 for 10 mins at room temperature followed by washing thrice with ice-cold PBS. Cells were further blocked in 1% (w/v) BSA in PBS containing 0.2% (v/v) Tween 20 for 30mins to block unspecific binding. Cells were incubated in primary antibodies (diluted in blocking solution) overnight at 4^0C in a humidified chamber followed by washing in PBS for 5mins thrice. They were then incubated in corresponding TRITC or FITC conjugated secondary antibodies (diluted in blocking solution) for 1hr at room temperature in dark and again washed in PBS three times for 5mins each. Coverslips were then mounted on slides in Anti-fade mounting medium with DAPI (4',6-diamidino-2-phenylindole) and sealed with nail polish to prevent desiccation. Slides were stored at -20°C until observation. Images were observed at 63X/40X objective in Zeiss confocal laser scanning LSM 510 microscope and analysed using LSM software.

2.8 BrdU Assay

BrdU is incorporated into the DNA of cells that are in the S-phase of the cell cycle. A short exposure time (2-4hrs) ensures few BrdU-containing cells to progress through the M-phase which minimizes the number of 'double-nuclei' and gives a more accurate quantification of cell proliferation.

To determine the number of proliferating cells, cultures were incubated with BrdU added to the culture medium at a final concentration of 3µg/ml for 4hrs. Media was removed and cells were fixed in 4% paraformaldehyde for 30-60 mins followed by 3 PBS washes for 5mins each. Fixed cultures were treated with 2N HCl for 30mins to separate DNA into single strands and again rinsed thrice with PBS. Cells were blocked for non-specific epitopes with 5% normal horse serum (in PBS with 0.2% Triton X-100) for 1-2hrs. BrdU incorporation was visualized by immunocytochemistry using mouse anti-BrdU primary antibody (diluted in the blocking solution) incubated at 4^0C overnight. Next day, cells were rinsed thrice with PBS followed by incubation in anti-mouse FITC secondary antibody (diluted in blocking solution) for 2hrs at room temperature in dark. Cells were mounted in anti-fading mounting medium containing DAPI nuclear dye. Fluorescent staining was imaged using the Floid cell imaging system (Thermo fisher scientific). The labeling index, corresponding to the ratio of BrdU+ cells to total cells, was determined from photomicrographs of individual fields of BrdU-labeled and DAPI-stained nuclei. A minimum of 30 BrdU+ cells were counted for each labeling condition.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Astrocytes were transfected with Negative control siRNA and MeCP2 siRNA and condition media was collected after 24hrs of treatment and frozen until use. Secreted BDNF and NGF protein levels in culture supernatants were measured using high sensitivity ELISA kit (Thermo Fisher). All antibodies and reagents were diluted according to the manufacturer's protocol.

Standards and samples were added in duplicates to antibody pre-coated 8-well strip and incubated overnight at 4^0C with gentle shaking. Next day the solution was

discarded, strips were washed 4 times with wash buffer and blotted against a blotting paper after the last wash to ensure complete removal of solution. 1X biotinylated antibody was added and incubated for 1 hour at room temperature followed by 4 washes. Streptavidin-HRP solution was subsequently added, incubated for 45 minutes at room temperature, again washed 4 times before adding the TMB substrate for 30 minutes and incubated in dark, followed by adding the stop solution. Strips were evaluated within 30 minutes of stopping the reaction. Absorbance was measured at 450nm on a microplate reader (Biotex instrument, ELX-800). Readings from the standards were used to create a standard curve, off which sample readings were read and concentration of neurotrophins was calculated in pg/ml. All samples were assayed in 3-4 separate ELISA assays, with two replicates per sample per ELISA strip.

2.10 MTT Assay

Cell survival was assessed by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], a colorimetric dye, that detects the formation of dark blue formazon product from MTT in active mitochondria. In brief, OPCs and neurons were treated with MeCP2 knockdown astrocyte condition media for 24 hours along with relevant controls. 1mg/ml MTT was added to the culture medium of these cells and incubated for 4hrs in dark at 37^0C in a humidified atmosphere with 5% CO_2. The culture medium containing MTT was removed and 150µl of DMSO was added to each well. The absorbance of the dissolved dye was measured at 490 nm. Data was expressed in OD units. Cells without any treatment were considered as blank.

2.11 Myelin extraction

Myelinating co-cultures of DRG neurons and oligodendrocytes grown on an astrocytic bed were established (as described in section 2.3) and myelin was extracted from these cultured cells (Thomson et al., 2008). The cellular material was scraped off the culture dish and re-suspended in chilled 50 mm HEPES (pH 7.4), supplemented with protease inhibitors. The cell lysate was passed through a syringe several times to form a cell suspension which was mixed with 0.85M sucrose solution to a total

volume of 2ml. The cell suspension was transferred to a centrifuge tube and gradually over-layered with 1ml of 0.25M sucrose solution. The resulting homogenate was subjected to ultracentrifugation at 75,000g for 1.5 hrs at 4^0C in a Beckman Coulter MLA-55 rotor. The myelin fraction was visible at the 0.85/0.25 sucrose interface which was gently harvested and transferred to another centrifuge tube. The resulting fraction was subjected to two rounds of hypotonic shock by addition of chilled distill water followed by centrifugation at 13,000g for 30 mins to pellet the myelin extract. The final pellet was resuspended in 50mm HEPES and protein concentration was quantified using Qubit 2.0 Flurometer.

2.12 Protein precipitation, In-gel digestion and MALDI-TOF

Trichloroacetic acid (TCA) protein precipitation: Proteins were precipitated from condition media of control and MeCP2 knockdown astrocytes using TCA precipitation method. Briefly, 1 volume of TCA was added to 4 volumes of condition media and incubated at 4^0C for 10 mins followed by centrifugation at 14,000rpm for 15 mins. Whitish and fluffy protein precipitate was subjected to 2 washes of acetone and centrifuged at 14000rpm for 5 mins. Protein pellet was air-dried to completely remove the acetone, boiled and re-suspended in 2X lysis buffer (for SDS-PAGE followed by in-gel digestion) or 200nM Tris buffer (for MALDI analysis)

SDS-PAGE: Equal protein was loaded (50µg) and resolved by SDS-PAGE on a 12.5% gel using a constant voltage of 100V. The gel was removed from glass plates and rinsed in distil water followed by incubating it in Coomassie stain overnight at room temperature. Next day, the gel was given 2-3 washes with distill water to completely remove the excess stain followed by incubating in destaining solution with gentle agitation. Destaining was done until the background was nearly clear and the bands were clearly visible.

In gel digestion: The entire gel slab was rinsed with water and kept on a plastic tray. Protein bands of interest were excised using a fresh scalpel. The excised bands were further cut into small cubes and transferred to a micro-centrifuge tube. Gel pieces cut from Coomassie stained gels were further destained using 100µl of 100mM ammonium bicarbonate: acetonitrile (ACN) (1:1 v/v) with occasional vortexing for 30

mins depending on the staining intensity. 500µl of neat ACN was added and incubated at room temperature with occasional vortexing until gel pieces become white and shrink in size followed by removal of ACN. At this point samples can be stored at -20^{0}C for a few weeks. Gel pieces were then treated with 50µl of 0.5mg/ml trypsin buffer by completely covering them and incubated at 4^{0}C for 30 mins and for another 90 mins at room temperature followed by addition of 10-20µl of 10mM ammonium bicarbonate buffer. Samples were briefly centrifuged to spin down the gel pieces and supernatant containing trypsin and ammonium bicarbonate was collected and stored at -20^{0}C for further analysis.

MALDI-TOF mass spectrometry: Total protein or in-gel digested peptides samples were mixed with α-Cyano-4-hydroxycinnamic acid (HCCA) matrix in different dilutions and dried on to a metal plate. The metal plate was then placed in a high vacuum source chamber in the mass spectrometer and samples were vaporized by blasts from a nitrogen laser. The emitted ions fly up to a mass analyser and their mass-to-charge ratio (m/z) was determined by the 'time-of-flight'. MALDI-TOF was carried out on Bruker Ultraflextreme machine at TIFR, Mumbai. Spectra analysis was done using FlexControl and FlexAnalysis softwares and auto-proteolysis products of trypsin were used as internal calibrates. Identification of proteins was performed using Mascot software (available at www. matrixscience.com).

2.13 Statistical Analysis

Results are expressed as mean±standard error mean (SEM) and differences between treatment groups were statistically analysed using student's t-test and one way analysis of variance (ANOVA) followed by Bonferroni's post hoc test wherever appropriate. Statistical analysis was done with Prism 6 software (GraphPad Software Inc.). A value of $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).