CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Isolation and Culture of primary Oligodendrocytes and Astrocytes

In order to obtain highly enriched rat glial cell culture (astrocytes and oligodendrocytes) previously described methods were used (Chen et al., 2007; Frost et al., 2009; O'Meara et al., 2011; Tripathi et al., 2017; Vora et al., 2011). P0-P2rat pups were used for the study and the animal protocols were approved by the Institutional Animal Ethical Committee (IAEC) and in line with CPCSEA guidelines. The rat pups were decapitated and their brains were isolated in ice cold Hank's balanced salt solution (HBSS). The ratcerebra from the brains, after having the meningeal layer removed were dissected, minced, and digested at 37°C with 0.8 ml of DNase-I stock solution (Sigma)with a concentration of 0.2 mg /ml and 0.6 ml of 0.25% trypsin stock solution (Invitrogen) in 13.6 ml HBSS, to generate a single-cell suspension. These cells were plated on poly-L-Lysine [(PLL)-Sigma] coated T75 cm2 flasks (Eppendorf) with high-glucose Dulbecco's modified Eagle's medium [(DMEM)-Gibco] containing 10% of heat-inactivated fetal bovine serum [(FBS-Gibco] and 1%penicillin/streptomycin (1X-Invitrogen) then incubated at 37ºC in the presence of 5%CO_2. This cell suspension was uniformly spread over the entire flask surface. For the period of next ten days, 10ml of DFG media (DMEM+antibiotic+FBS) was replaced in the flasks after an interval of every 2-3 days to obtain a mixed glial cell culture. Oligodendrocyte precursor cells (OPCs) are present as oligo-spheres on an astrocytic monolayer. These oligodendrocytes (OLGs) are further isolated by shaking off them overnight in an orbital shaker for 18-20h at 37°C at a speed of 220rpm. Further, pure OPCs were isolated, based on differential adhesion, on a non-tissue culture plastic petri dish for 1h in the CO_2 incubator to remove the microglial and astrocytic cells. This cell suspension was passed through 40µm pore size cell strainer (Corning) and collected in a 50ml falcon tube (Corning) for centrifugation at 100g for 10 min. After discarding the supernatant, cell pellet was dissociated in 0.5ml of the remaining medium. Using a hemocytometer, colourless viable cells were counted by Trypan blue exclusion assay. DMEM+B27 Media (Invitrogen) was used to dilute these

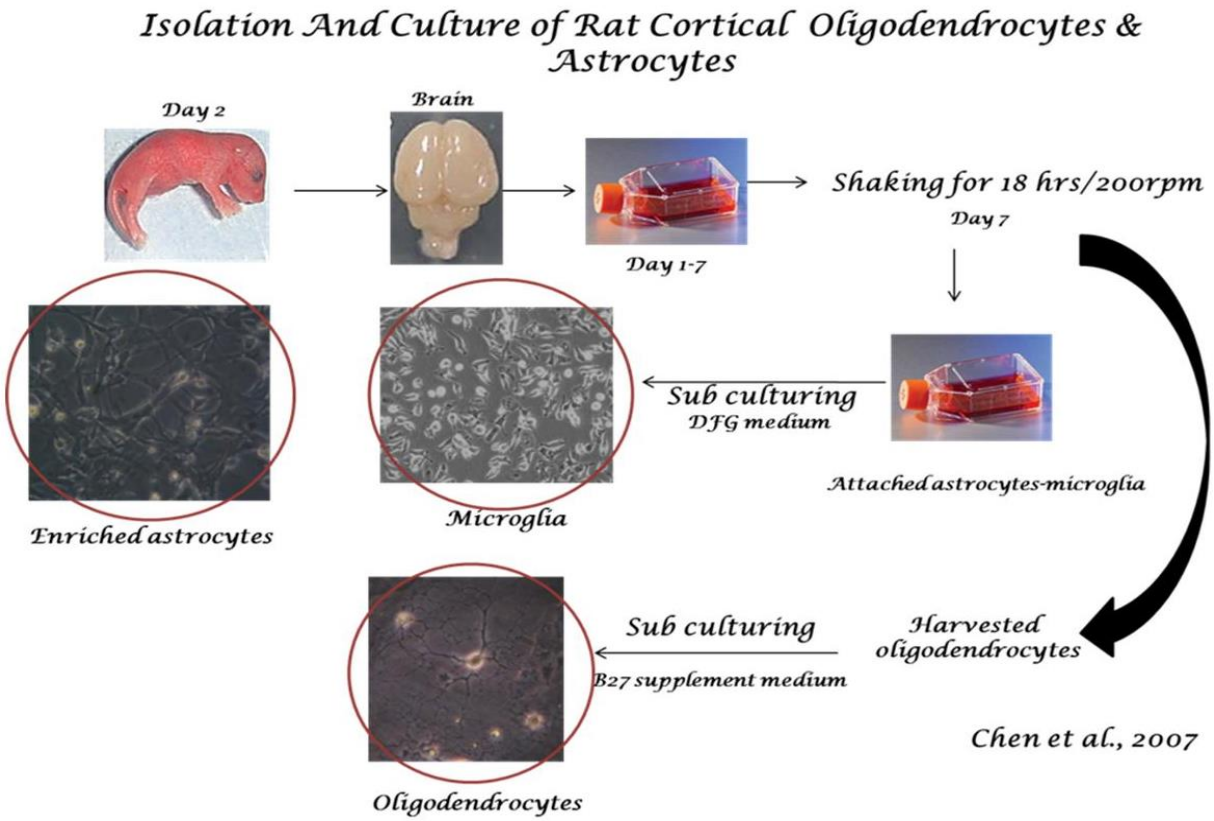

Fig. 2.1 Flow diagram of isolation and culture of OLGs and Astrocytes

oligodendrocyte progenitor cells to obtain desired cell number. OPCs were cultured in B27 medium (DMEM+B27 supplement) containing PDGF-AA (Sigma) (10ng/ml of Sato media) for proliferation of OPCs into OLGs. OPCs were grown in Sato medium (Bottenstein & Gordon, 1979) without PDGF-AA (Sigma) for differentiation of OPCs into OLGs. The cultures were characterized by immunostaining using stage specific markers of OLG development which showed 95% purity of OLGs. Astrocytes upon reaching confluency, were trypsinized and replated. Cells were used after the fourth passage (P4) in all the experiments, and were seeded at $1x10^5$ cells in 6-well plate dishes. Cultures were assayed by immunochemical analysis using antibodies against GFAP in order to determine the degree of enrichment; the astrocyte cultures were early pure without contamination of microglia or neurons (Refer chapter 3).Figure 2.1 shows the flow chart of derivation of OLGs and astrocytes from P0-P2 day rat pup. Use, care and housing of all animals were done in compliance with Institutional Animal Ethical committee (IAEC), The Maharaja Sayajirao University of Baroda. The protocol numbers

are mentioned as follows: ZD/13/2014, ZD/28/2014, BC/13/2015, ZD/03/2016, ZD/03/2017.

2.2 Glioma Cell line

The Rat C6 glioma cell line was procured from NCCS (National Centre for Cell Science, Pune, India Rat C6 glioma cells were grown as monolayer cultures in DMEM (GIBCO)supplemented with 10% FBS and penicillin/streptomycin (Invitrogen). Cell line was maintained at 37°C in 5% CO2.

2.3 N19 Cell line

The mouse oligodendroglial cell line N19 was a kind gift from Dr. Pablo Paez (Department of Pharmacology and Toxicology, Hunter James Kelly Research Institute, School of Medicine and Biomedical Sciences, SUNY, University at Buffalo, NYS Center of Excellence, 701 Ellicott St., Buffalo, NewYork, USA). These cells were conditionally immortalized OLGs. The N19 OLGs were grown up to 70% confluency at 36 °C, in 75-cm flasks (2–3 × 10^6 cells/flasks) or PLL coated coverslips (2 × 10^5 cells/ml; for immunocytochemical studies) in Dulbecco's modified Eagle's medium (DMEM)/F12 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Silvestroff et al., 2013). For the differentiation studies, the N19 cells were placed at 39°C in differentiating medium DMEM/F12/1% FBS and 1% penicillin/streptomycin with apotransferrin (100 μg/ml) for 7 days (Paez et al., 2004).

2.4 Immunocytochemistry (ICC)

Cells were seeded on the coverslips in 12 well plates and were allowed to grow in the complete (DFG) media. Control and Treated cells were fixed in 4% paraformaldehyde (pH 7.4) for 10 min at room temperature (RT) and were washed with ice cold phosphate buffered saline (PBS) pH7.4for three times. Next, the cells were incubated in 0.25%

Triton X-100 in PBS for 10 min at RT for permeabilization. Cells were washed for three times with ice cold PBS for 5 min each. For blocking unspecific binding of antibodies, cells were incubated with 1% BSA in PBS-T (0.1% Tween-20 in PBS) for 30 minutes. Then the cells were subjected to diluted primary antibody incubation (diluted in 1% BSA in PBST) overnight at 4ºC in a moisturized chamber and were washed three times in PBS for 5 min each. Then the cells were incubated with secondary antibody (in 1% blocking buffer) which was tagged with fluorophore for 1h at RT in the dark and were washed three times with PBS for 5 min each. For mounting, the coverslips were treated by using Gold Antifade Mountant (Invitrogen) with DAPI (4',6-Diamidino-2-phenylindole) and were sealed with transparent nail polish to prevent drying. Slides were stored until use at -20°C. Images were analysed through confocal microscopy. The antibodies used in the immunostaining are listed in Table 2.1.

Table 2.1 Antibodies for Immunofluorescence

Antibody	Dilution	Purpose	Manufacturer
PDGFRα	1:50	OPC Marker	Santacruz
O4	1:100	Immature OLG marker	R&D
MBP	1:100	Mature OLG marker	Abcam
GFAP	1:100	Astrocyte Marker	Santacruz
MeCP2	1:50	Nuclear Protein	Abcam
pS80 MeCP2	1:50	Nuclear Protein	ECM Biosciences
pS421 MeCP2	1:50	Nuclear Protein	ECM Biosciences
Anti-Rabbit FITC	1:200	Secondary Antibody	Abcam
Anti-Goat TRITC	1:200	Secondary Antibody	Abcam
Anti-Mouse TRITC	1:100	Secondary Antibody	Abcam

2.5 Sub-Cellular fractionation

The nuclear and cytosolic fractions were prepared using the NE-PER kit (from Thermo) as per the manufacturer's instructions. Briefly the cells were seeded in 6 well plates at a seeding density of 0.3 x 10^6cells. After reaching confluency, the cells were serum starved for 24h, and thenthe cells were then treated with growth factors and ECMs as mentioned in the section 2.10.After the treatment, the media was discarded and cells were harvested with trypsin-EDTA (Invitrogen) and then centrifuged at 500×g for 5min. Further, the cells were washed by resuspending the cell pellet in PBS. The cells were then transferred to a 1.5mlmicrocentrifuge tube and pelleted by centrifugation at 500×g for 2-3 min. The supernatant was carefully removed and discarded. Ice-cold Cytoplasmic Extraction Reagent I (CER I) was added to the cell pellet using the reagent volumes indicated in the kit. After adding CER-I, the tube was vortexed vigorously on the highest setting for 15 seconds to fully resuspend the cell pellet followed by 10min of incubation on ice. After that ice-cold Cytoplasmic Extraction Reagent II (CER II)was added to the tube and the tube was vortexed for 5 seconds on the highest setting followed by incubation of 1min on ice. Again by vortexing the tube for 5 seconds on the highest setting, the fractions were separated by centrifugation for5min at maximum speed in a microcentrifuge (~16,000 × g) at 4°C. The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube. The insoluble (pellet) fraction was suspended in ice-cold Nuclear Extraction Reagent (NER) and vortexed on the highest setting for 15 seconds and this was repeated every 10 minutes, for a total of 40 minutes by incubating the samples on ice. The tubes were centrifuged at maximum speed (~16,000 × g) for 10 minutes at 4. The supernatant (nuclear extract) was immediately transferred to a clean pre-chilled tube. The nuclear and cytoplasmic fractions were stored at -20°C until used. Figure 2.2 shows the flow diagram of the sub-cellular fractionation. The proteins were analysed using immunoblotting (Section 2.7).

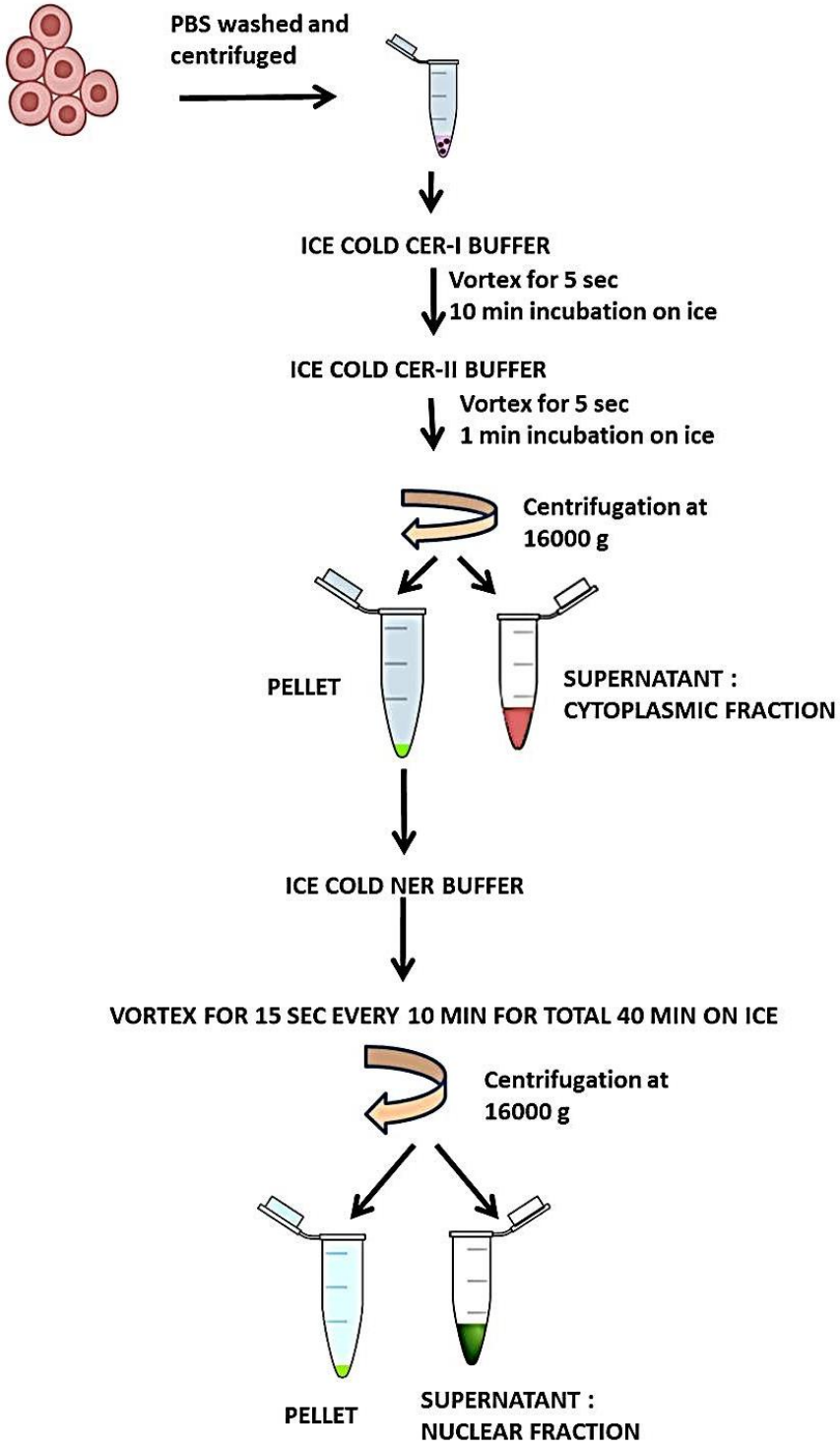

Fig. 2.2 Flow diagram of sub-cellular fractionation

2.6 Chromatin fractionation

Nuclei isolation: After the glial cell treatment for the mentioned time, media was discarded and cells were washed with 2 ml of chilled PBS at 4^0C. Cells were fixed with 1% formaldehyde and were incubated at RT for 10 min. 10X glycine was added to quench excess formaldehyde and was swirled for 5 min at RT. Cells were washed with PBS and were centrifuged for 15 min at 800×g at 4^0C. Pellet was resuspended in nuclei isolation buffer and was incubated on ice for 15 min, and was vortexed for 10 seconds in every 5 min. The cells were centrifuged for 5 min at 800×g at 4^0C. Pellet was resuspended in 200µl of nuclear buffer (20mM Tris-HCl [pH 7.5], 70mMNaCl, 20mMKCl, 5mM MgCl2, and 3mM CaCl2 supplemented with protease inhibitor cocktail). Isolated Nuclei were stored at -20^0C until use.

Nuclear Fractionation: The micrococcal nuclease fraction of the nuclei of glial cells was performed as previously reported (Gonzales et al., 2012). The nuclei were isolated from cells as described above by using the kit. This nuclear suspension was subjected to digestion with 3U of micrococcal nuclease (Sigma) at RT for 12min. The digestion was terminated by the addition of EDTA and EGTA 5mMeach; then centrifuged at 5,000×g for 3 min at 4^0C, and the supernatant was designated the S1fraction. The pellet was then resuspended in 2mM EDTA for 15 min at 4°C followed by centrifugation, and the supernatant and the pellet were designated the S2 and P fractions respectively. The fractions were treated with lysis buffer (50mMTris-HCl [pH 7.5], 100mMNaCl, 5mMEDTA, 0.5% SDS) for 1 h at 37°C. Aliquotes were taken from these chromatin fractions S1, S2, and P and were subjected tophenol-CHCl3 extraction and analysed on agarose gel electrophoresis for digestion. Proteins were analysed by SDS-PAGE. Fig-2.3 shows the flow diagram of the chromatin fractionation.

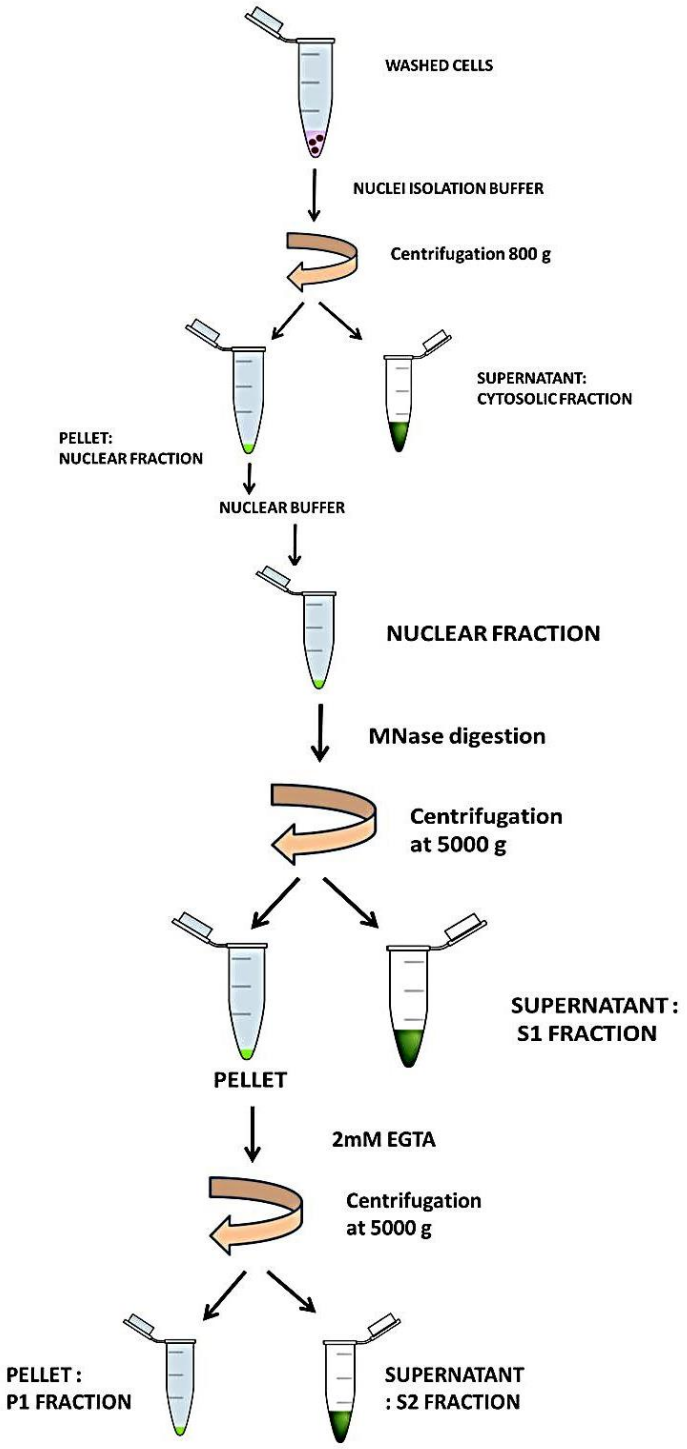

Fig. 2.3 Flow diagram of chromatin fractionation

2.7 Protein Extraction, Protein Quantification and Western Blotting

Control and treated cells were scraped and collected in microcentrifuge tubes for centrifugation at 5000rpm for 5 min at 4°C. The pellet obtained was washed twice with ice cold PBS. Based on the cell density, 100-200µl of RIPA lysis buffer (10mM Tris-Cl pH 8.0, 1mM EDTA, 0.5mM EGTA, 1% triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl) along with the protease inhibitor cocktail (Roche)was added to lyse the cells. These cell lysates were stored at -20ºC until use. Further the total protein was quantified using Qubit protein assay kit (Invitrogen) in Qubit 2.0 fluorometer (Invitrogen). To ready the samples for Western blotting, 40µg of total estimated protein, from each sample were aliquoted to which 5x loading dye and reducing agent were added. These samples were mixed and incubated at 95°C in water bath for 5 minutes following a quick spin in the pulse spin centrifuge. Proteins were allowed to resolve by SDS polyacrylamide gel electrophoresis (10%) at constant voltage (100V) for 90 minutes and Thermo PAGERULER Plus Ladder was used as a molecular weight marker. Following electrophoresis, gel was removed to be placed in 1x Transfer buffer (0.190mM glycine, 25µM tris-base, 200ml/l methanol) at room temperature. A sandwich of transfer sponge, filter paper, SDS gel, 0.2µM nitrocellulose membrane, filter paper, and a transfer sponge was prepared and clamped in a cassette from anode to cathode side for electroblotting at 100 volts for 90 minutes in a Bio-Rad Mini Trans-Blot apparatus containing transfer buffer and ice pack.

After the transfer for blocking nonspecific antibody binding, this nitrocellulose membrane was placed in clean plastic container to be with 5% BSA (Bovine Serum Albumin) in TBS-T (20mM Tris, 500mM NaCl and 0.05% v/v Tween 20) and kept on an orbital shaker at room temperature for 60 min. After blocking, the blots were incubated at 4°C overnight on orbital shaker with primary antibody (made in 5ml of 5% BSA). Concentrations of all primary antibodies used can be found in Table 2.2.Post incubation with primary antibody, washing of the blots was done with TBS-T for 6 times, each for 10 minutes on the orbital shaker. This blot was then incubated for 1h at room temperature on the orbital shaker in 5ml of secondary antibody conjugated with horseradish peroxidase (HRP) in 10mL of 5% BSA solution (dilutions found in Table 2.2).This was followed by 6 times of washing with TBS-T for 10 min each. Further blots were developed using specific immunoreactivity and visualized using an ECL kit (Invitrogen). In the dark room, X-ray cassette was opened and film was placed inside for

exposure. Separate pieces of film were exposed for 30 seconds, 2 min, 5min and 8min. The film was developed and the immunoreactive bands were digitally scanned quantitated by densitometry using ImageJ.

2.8 Co-Immunoprecipitation

For co-immunoprecipitation, N19 OLGs were washed twice with ice cold PBS and lysed in lysis buffer (150mM NaCl, 20mM Tris-HCl, 1.5mM MgCl2, 1% NP-40,15% glycerol,2mM EDTA) by a 30-min incubation at 4 °C, containing0.5% bovine serum albumin and proteinase inhibitor cocktail(Sigma). Equal amounts of clarified cell lysates were incubated with 5µg of anti-α6 (R&Dsystems) antibody overnight at 4°C. After adding protein A/G agarose beads (Santa Cruz) for 1 h, the suspension was centrifuged, and pellets were washed three times with lysis buffer, and one time with PBS. The immunoprecipitated proteins were solubilized in Laemmli lysis buffer, resolved by SDS polyacrylamidegel electrophoresis, and blotted with antibodies to integrin α6 (R&D systems) and β1 (Thermo Fisher) subunits. Bound antibodies were revealed by horseradish peroxidise conjugated secondary antibodies and the ECL detection system.

2.9 Microscopy, quantification and statistical analysis

Confocal microscopy images were analysed by ZEN 2012 imaging software. For statistical analysis, a minimum of 150randomly chosen cells per condition were analysed (N=3independent experiments with 3–4 replicates). Differences between the treatment groups were analyzed using Student's t test, or one way analysis of variance with Bonferroni's post-test where appropriate. Statistical analysis was performed with Prism 3software (GraphPad Software Inc.). The value of $P<0.05$ was considered significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

Table.2.2 Antibodies for Immunoblotting

Antibody	Dilution	Purpose	Manufacturer
MeCP2	1:1000	Epigentic Regulator	Santacruz
β-Actin	1:2000	Housekeeping gene	Sigma
GAPDH	1:2000	Cytoplasmic Marker	Sigma
pAKT	1:1000	Signaling Protein	CST
AKT	1:1000	Signaling Protein	CST
Total ERK ½	1:1000	Signaling Protein	Abcam
pERK1/2	1:1000	Signaling Protein	Santacruz
pS80 MeCP2	1:1000	Phosphorylated MeCP2	Abcam
pS421 MeCP2	1:1000	Phosphorylated MeCP2	Abcam
Histone H3	1:1000	Nulear Protein Marker	Invitrogen
Anti-Rabbit HRP	1:200	Secondary Antibody	Sigma
Anti-Goat HRP	1:200	Secondary Antibody	Sgma
Anti-Mouse HRP	1:100	Secondary Antibody	Sigma

2.10 Cell Treatments

2.10.1 Growth Factor and ECM treatments

Glial cells were plated in DMEM/F12and 1% penicillin/streptomycin (Gibco) on poly-L-lysine (PLL; Sigma)-coated dishes or glass coverslips, and were serum starved overnight prior to any treatment. Cells were exposed to the factors mentioned in the following Table 2.3 for 1 h. LPS treatment was given for 4h to the astrocytes. PLL was used as control substratum which supports cell adhesion and spreading without engaging integrins.

Table.2.3 Factors used for Cell treatments

Factors	CNS Glial cell type	Concentration
Growth factors	PDGF	10ng/ml
	FGF	10ng/ml
Extracellular matrix	FN	10µg/ml
	LN	10µg/ml
Neurotrophins BDNF & NGF	BDNF	50ng/ml
	NGF	50ng/ml
Inflammatory Factor	LPS	100ng/ml

2.10.2 Inhibitors/blockers

For the inhibition studies of signalling pathways and membrane receptors following inhibitors/blockers were used (Table2.4). Concentration and exposure time were determined using previous studies. When treating with a cell signalling inhibitor; the inhibitor was added to the cultures 1hr prior to the treatment. For blocking studies of integrin, blocking antibody was added 1h prior to the experiment.

Table.2.4 Antagonists and Concentration for Cell treatments

Antagonist/Agonist/ Inhibitor	Concentration	Function	Manufacturer
UO126	10µM	MAPK/ERK inhibitor	EMD
LY290042	50µM	Selective inhibitor of PI3K pathway	EMD
KN93	5µM	Inhibitor of CamKinaseII	EMD
K252a	0.2µM	Inhibitor of TrkB receptor	Tocris
Blocking antibody α6β1	1:100	Block the α6β1 integrin	R&D