2. MATERIALS AND METHODS

2.1: Neural stem cells culture: Neurosphere generation:

Neural stem cells culture was established from postnatal day 0-1 (P0-1 day) rat pups following Pacey et al. Protocol (Laura Pacey *et al.,* 2006) with minor modifications. Brain cerebral cortices were dissected out and meninges were carefully removed from the cortices and finally transferred to a 60 mm non-tissue culture treated culture dish with HBSS (Gibco-Invitorgen). The entire cortex was used to generate neurosphere culture. (Alternatively, the entire brain from a rat pup (less than 2 days old) can be used to generate maximum number of neurospheres). Cortex was minced into smaller pieces, taken into a 15ml tube and centrifuged for a 5 minutes at 250g. The supernatant with HBSS was removed and 1X TPVG (Invitrogen) was added to the pelleted tissue followed by incubation at 37^0C in a water bath for 10 - 15 minutes with regular trituration every 5 minutes to check the tissue dissociation. The tissue suspension was then centrifuged for 5 minutes at 250g and supernatant containing TPVG was completely removed followed by addition of DMEM:F12 (Gibco-Invitorgen). The digested tissue was gradually triturated to break the tissue into a nearly single cell suspension. The homogenate was then centrifuged for 5 minutes at 500g, supernatant was removed, pelleted cells were re-suspended in Serum free neural stem cells medium (composition of media as described below) and plated on non tissue culture treated T25 flask (Corning). The culture flasks were incubated at 37°C with 5% CO2 for further experiments. Cells were allowed to form colony of neurosphere till 5-7 days in suspension culture.

Neural stem Cell Media: DMEM F12 (Gibco) with 1x B27supplement (50X without insulin Gibco 05-0129SA); Fibroblast growth factor-basic (FGF) (Sigma cat. no. F0291) – Final concentration: 20 ng/ml; Epidermal growth factor (EGF) (Sigma cat. no. E4269) – Final concentration: 20 ng/ml; L-Glutamine -- Final concentration: 2mM and Insulin -- Final concentration: 0.22mM

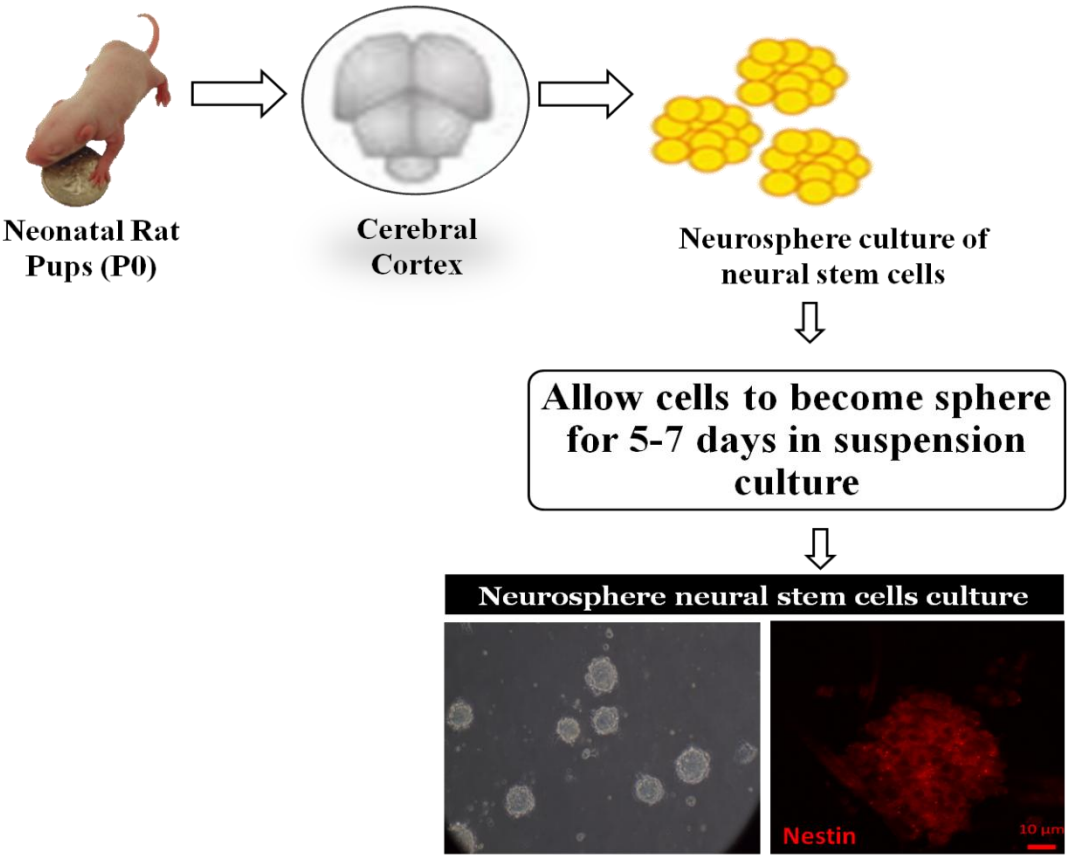


Figure 2.1: Diagrammatic representation of neural stem cells from rat pups brain.

2.2: Glial cells: Astrocytes and Oligodendrocytes progenitor cells (OPCs); isolation and culture:

Mixed glial cells were isolated and cultured from cerebral cortices of P0-P2 day rat pups (Chen *et al.*, 2007). Briefly, the cerebral cortex were dissected, cut into smaller pieces and treated with DNase I and trypsin in HBSS at 37°C, to generate a single-cell suspension. Dissociated cells were plated on PLL coated T-75 cm^2 tissue culture flasks in DFG media (DMEM + 10%FBS + 1% penicillin/streptomycin) and incubated at 37^0C in a CO_2incubator for 10 days. Culture media was changed every third day. After a week, mixed glial cultures are observed with microglia and OPCs growing on an astrocytic bed. 10 days post culturing, flasks were sealed and shaken on an orbital shaker at 150rpm for 1hr to remove microglia. Media in flasks was replenished and shaken again at 200rpm for

18-20 hrs at 37^0C to separate OPCs from an astrocytic monolayer. The cell suspension was passed through 40µm pore size filter and the collected suspension was centrifuged at 500g for 10mins. Cell pellet was dissociated in DMEM medium and cultured in Sato medium (Bottenstein & Sato, 1979) .Underlying cultures of astrocytes were dissociated with 0.1% trypsin and passaged using DFG medium for further experiments.

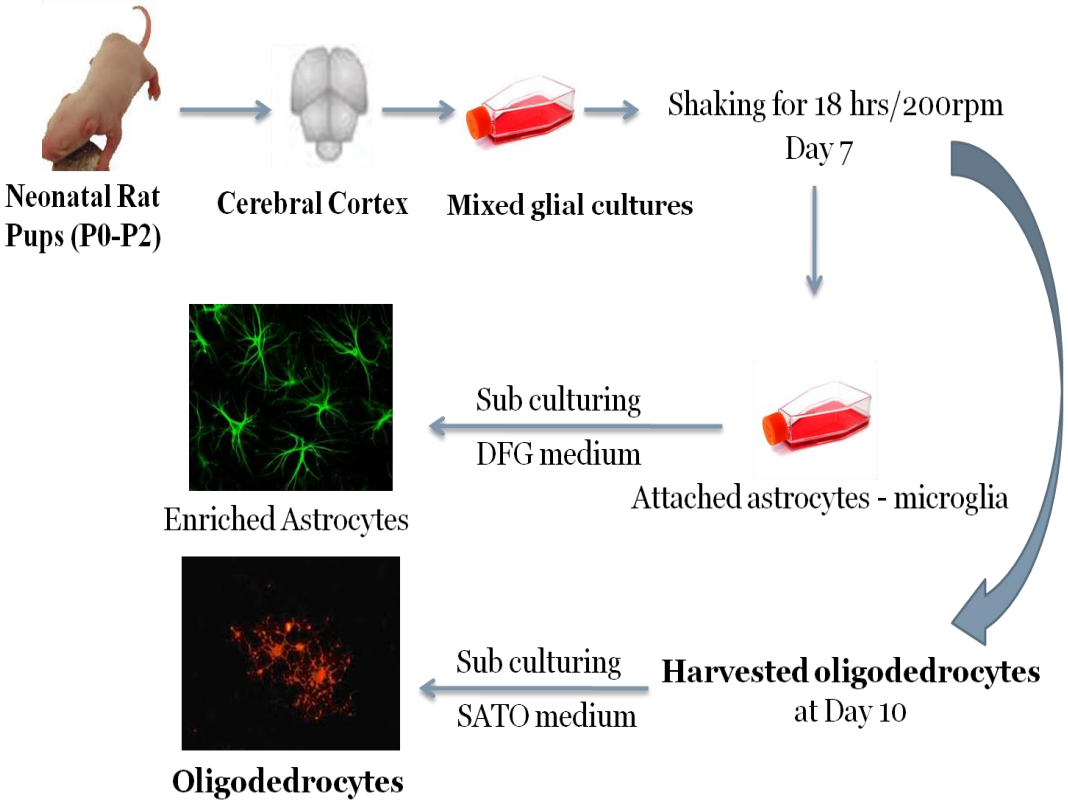


Figure 2.2: Schematic diagram of glial cells isolation and culture: Astrocytes and Oligodendrocytes progenitor cells

2.3: Myelinating Spinal Cord Culture:

Myelinating Spinal Cord culture was established from E13.5 time pregnant mice following Thomson et al. protocol (Thomson *et al.*, 2008). Wild-type balb/c female mice were time-mated and embryos were collected on embryonic day E13.5. The day of plugging was denoted as E0.5 and the fetuses were used for culture at 13 days later. At the appropriate gestation stage, the pregnant mouse was sacrificed by cervical

dislocation. The gravid uterus was removed aseptically into a sterile Petri dish containing HBSS, the embryos were extracted and the umbilical cord was cut. Fetuses were decapitated at about 3mm rostral to the cervical flexure thus including the caudal part of the myelencephalon (which increases the tissue amount for cell harvesting) along with 8-10mm of spinal cord which was transferred to sterile DMEM (Gibco-Invitrogen). Remaining meninges were removed, tissue was minced to small pieces and transferred to a tube containing 1mL of HBSS (calcium-magnesium free), 1X trypsin and 1% collagenase and incubated at 37^0C for 20 mins.

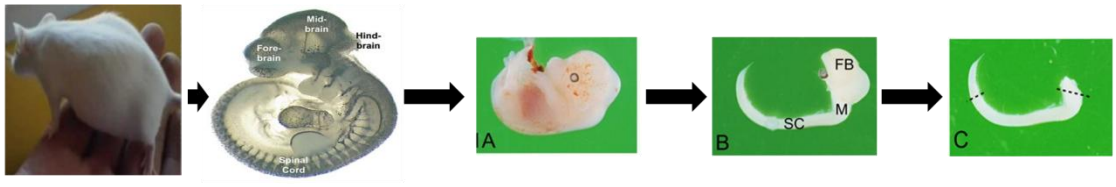
Post incubation, SD solution (Recipe as mentioned below) was added and tissue fragments were dissociated by gentle trituration. 5mL of Plating medium (PM, recipe as mentioned below) was added into this and the cells were pelleted by centrifugation at 500g. Supernatant was discarded; cells were re-suspended in fresh PM media and plated in tissue cultures plates for incubation at 37^0C for 2-3 hrs. Post incubation, cells were fed with DfM:PM (1:1 ratio). Cultures were fed thrice per week by removing half of the medium and adding fresh DfM medium; with insulin for first 12-13 days and without insulin for the remaining time. In myelinating spinal cord culture myelination occurs in autonomously, at Day in-vitro 15 (DIV 15) myelination event starts and gets completed at DIV 23. At DIV 23 mostly all the neurons gets myelinated thus, this time window was selected for remyelination study in our present work.

Media Preparation:

- **SD solution:** 0.52 mg/mL soybean trypsin inhibitor, 0.04 mg/mL bovine pancreas DNase and 3.0 mg/mL BSA fraction V made up in Leibovitz's L15 medium.
- **Plating medium (PM):**50% DMEM, 25% horse serum, 25% HBSS (with calcium and magnesium), and 2 mm glutamine.
- **Differentiation medium (DfM):** DMEM, 0.5% hormone mix, 10 ng/mL biotin and 50nM hydrocortisone.

  Hormone mix: N2 mix- 1 mg/mL apo-transferrin, 20mm putrescine, 4µM progesterone, and 6µM selenium.

  Insulin was added to the DfM for the first 12–14 days of culture only.



Dissection of the neuraxis from embryonic day 13.5 time-mated mouse embryos.
(A) Intact embryo removed from uterus.
(B) Dissected neuraxis left intact for orientation. Forebrain (FB); Myelencephalon (M); Spinal cord (SC).
(C) Isolated myelencephalon–spinal cord as dissected during culture preparation. The dotted lines indicate the portion of myelencephalon and spinal cord used in the cultures. Ruler gradations are in millimetres.

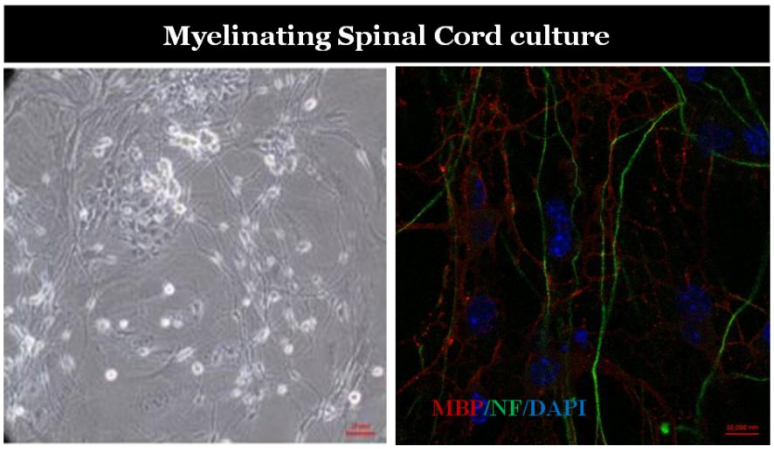


Figure 2.3: Diagrammatic representation of Myelinating spinal cord culture from E13.5 mice

2.4: Animal ethical approval:

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

2.5: Immunocytochemistry (ICC)

Cells were grown on PLL coated coverslips and fixed in cold 4% paraformaldehyde (PFA) for 10 minutes at room temperature followed by permeabilization in PBS containing 0.25% Triton X-100 for 10 minutes at room temperature. Cells were then blocked in 1% BSA (in PBS containing 0.2% Tween 20) for 30 minutes to block unspecific binding and incubated in primary antibodies (diluted in blocking solution)

overnight at 4^0C in a humidified chamber. They were then incubated in corresponding TRITC or FITC conjugated secondary antibodies for 1hr at room temperature in dark and mounted on slides in Anti-fade mounting medium containing DAPI. Details of all antibodies is provided in Table 1. Cells were then subjected for confocal microscopy. Images were observed at 63X/40X objective in Zeiss confocal laser scanning LSM 510 microscope and analysed using LSM software.

Table 1: Antibodies for immunoflorescence

Antibody	Dilution	Purpose	Company
Nestin	1:100	Stem cells marker	Santacruz
PDGFR-α	1:50	OPC marker	Santacruz
GFAP	1:200	Astrocytes marker	Abcam
Tuj 1	1:200	Neuron marker	Santacruz
NF	1:200	Neuron marker	Abcam
O4	1:100	Immature OLG marker	R & D system
MBP	1:100	Mature OLG marker and myelination marker	Abcam
BrdU	1:100	Proliferating cells marker	Pierce
Anti – Rabbit TRITC	1:200	Secondary Antibody	Genei
Anti – Goat TRITC	1:100 – 1:200	Secondary Antibody	Abcam
Anti- Goat FITC	1:400	Secondary Antibody	Genei
Anti – Mouse FITC	1:200 - 1:400	Secondary Antibody	Genei
Anti- Mouse TRITC	1:200	Secondary Antibody	Abcam

2.6: Polymerase chain reaction (PCR):

To study the PCR firstly total RNA was isolated using Trizol reagent (Invitrogen) and quantified in Qubit 2.0 Fluorometer using Qubit RNA assay kit. 1µg of total RNA was used for cDNA preparation by reverse transcription (RT) reaction using cDNA synthesis kit (Thermo scientific). cDNA was further used for the gene expression study by semi-quantitative PCR as well as Real time PCR.

Semi-quantitative PCR:

For semi-quantitative PCR, cDNA was suitably diluted and amplified with Titanium *Taq* polymerase with oligo (dT) and specific gene primers in a 20µl reaction mixture.PCR products were separated on 2% agarose horizontal gel and viewed with Bio-rad gel documentation instrument. Densitometry analysis of gel image was carried out by ImageJ software.

Real time PCR:

Quantitative RT-PCR was performed using SYBR Select Master Mix and specific gene primers in QuantStudio12K (Life Technology) real-time PCR machine. Specificity of products was confirmed by the melting curve of each sample. Data were normalized to the internal control GAPDH and analyzed using $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Primers used are listed in Table 2.

Table 2: Primers for PCR

Gene	Forward sequence	Reverse sequence	Reference
gapdh	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT	(Swiss *et al.*, 2011)
notch	CAATGGCACAGGGGCTATGA	TTAGCGGGTTGTACTGGCTG	(Wang *et al.*, 2017)
mbp	CTCTGGCAAGGACTCACACAC	TCTGCTGAGGGACAGGCCTCTC	(Paintlia *et al.*, 2004)
ngf	CCCCGAATCCTGTAGAGA	CACGCAGGCTGTATCTAT	(Aiga *et al.*, 2006)

bdnf	CCATAAGGACGCGG ACTTGT	GAGGCTCCAAAGGC ACTTGA	(Fuchikami *et al.*, 2009)

2.7: SDS-PAGE and Western Blotting:

Whole cell protein lysate was prepared in 2X lysis buffer with protease inhibitor and stored at -20^{0}C till further use. Protein quantification was carried out using Qubit protein assay kit in Qubit 2.0 fluorometer.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 5 mins and subjected to a pulse spin. Lysates of equal protein load (40µg) were resolved on a 10% SDS-PAGE gel and separated by electrophoresis at a constant voltage of 100V. After the run was complete, gel was equilibrated in 1X transfer buffer for 5 mins followed by transferring onto a nitrocellulose membrane. Transfer was carried out in a transfer unit for 90 mins at 100V. Transferred proteins were visualised by staining the membrane with Ponceau stain to check the quality of transfer. Protein blot was then blocked in blocking buffer (3% BSA in TBS-T) for 1 hr at room temperature followed by incubation in primary antibodies at 4^{0}C overnight. Details of primary antibodies are listed in Table 3. After primary antibody incubation, membrane was washed thrice in washing buffer for 10 minutes each, followed by incubation for 1 hour in corresponding horseradish peroxidase (HRP) conjugated secondary antibodies. Specific immune-reactivity was visualized using ECL kit (Bio-rad). Protein bands were imaged manually on X-ray film or observed digitally in chemidoc.

Table 3: Antibodies for immuneblotting

Antibody	Dilution	Purpose	Company
β- actin	1:1000	Housekeeping Protein	Santacruz
MBP	1:700	Myelin protein	Abcam
Tuj 1	1:700	Neuronal protein	Santacruz
GFAP	1:800	Astrocytic protein	Abcam
p-ERK	1:650	Signalling protein	Santacruz

Goat HRP	1:2000	Secondary Antibody- HRP conjugate	sigma
Mouse HRP	1:2000	Secondary Antibody- HRP conjugate	sigma
Rabbit HRP	1:2500	Secondary Antibody- HRP conjugate	sigma

2.8: Cell viability assay (MTT Assay):

Cell viability was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Riss *et al.*, 2016) which depends on the cellular conversion of tetrazolium salt into formazan product which can be detected using a micro well plate reader. Approximately, $2x10^3$cells/well were seeded onto 96 well plates and treated with treatment groups with relevant controls for 24 hours. 5mg/ml MTT was added to the culture media and incubated for 4 hrs at 37^0C. Later, the media was removed and 150µl of DMSO was added to all wells and mixed thoroughly to dissolve the dark blue formazan crystals. The absorbance of the dissolved dye was measured at 570-630nm. Data was expressed in optical density (OD) units which were plotted against control taken as 100% survival. All experiments were performed intriplicate and repeatedat least three times.

2.9: Cell Proliferation Assay (BrdU Assay):

To study the proliferation of the cells, BrdU assay was performed. For this assay cells were incubated with BrdU solution for 4 hrs at a final concentration of 3µg/ml. Media was removed and cells were fixed in 4% paraformaldehyde for 30 minutes followed by treatment with 2N HCl for 30 minutes to separate DNA into single strands. Cells were incubated with 5% normal horse serum for 1 hour for blocking at room temperature and followed by anti-BrdU primary antibody incubation for overnight at 4^0C. Following day, cells were incubated in FITC conjugated secondary antibody for 2 hours at room temperature. Further for mounting, anti-fade mounting medium containing DAPI was added to the treated cells. Finally, Fluorescent staining was captured using Floid cell imaging system (Thermo fisher scientific).The labelling index, corresponding to the ratio of Proliferated cells (BrdU+ cells) to DAPI positive cells (total cells), were determined

from photomicrographs of individual fields of BrdU-labelled and DAPI-stained nuclei. Ration of BrdU+ cells and DAPI+ cells was generated by GraphPad prism software in form of bar graph.

2.10: Cell migration assay (Scratch assay)

Cell migration was examined using scratch assay (Liang *et al.*, 2007). A scratch was introduced by scraping confluent cell monolayer with a sterile 200 µl pipette tip and then washed gently twice with cell culture medium to remove cell debris. Migrations of cells were monitored after 24, 48 and 72 hrs under inverted phase contrast microscope and percent migration rate (MR %) was calculated as: MR % = (A-B/A) X 100 %, where A represents the mean width of the cell scratch at 0 hr and B represents the mean width of the cell scratch after 24,48 and 72 hrs. Distance migration was measured using *NIS software* (Elements microscope imaging software).

2.11: NSC Differentiation Assay:

After third passage, cells were seeded at a density of 2 x 10^5 per 500 µL in a 12-well plate. To allow differentiation, culture wells were pre-coated with Poly-L-lysine (PLL; 10 mg/mL, Sigma). NSCs were fed by renewing the medium (DMEM/ F12 without FGF and EGF) every three days till 10 days to allow differentiation. Each plate contained a control consisting of NSCs not exposed to any added differentiation factors. The factors and concentrations tested were as follow: BDNF (10 and 50 ng/ml) and NGF (10 and 50 ng/ml) (Liu *et al.*, 2014, Ahmed *et al.*, 1995). Cells were allowed to differentiate for 10 days. Media composition for differentiation assay was DMEM/F12, 2% B27, 1% FBS, BDNF/NGF- 10ng/ml and 50ng/ml.

2.12: Morphometric analysis of Neurosphere:

Neurosphere number counting

Neurospheres were counted after the treatment of NGF and BDNF with and without Trk receptors inhibitors for 72 hours using phase contras microscope. Microscopy

observation gave total number of neurosphere colony and graph was made for treatment group against number of neurosphere, relative to control.

Neurosphere size assay

Neurosphere were treated with treatment groups morphological analysis, in regards to size of colony, was done through phase contras microscopy. Size of neurosphere (µm) was measured using *NIS software* (Elements microscope imaging software) at three different time points: 24, 48 and 72 hours.

2.13: Myelin extraction:

A lipid-rich fraction called myelin fractions was extracted from myelinating cultures (Thomson *et al.*, 2008). This myelin fraction was extracted from the cultured cells using a modified protocol for extracting myelin from CNS tissue (Yool *et al.*, 2001).Briefly 100 µL of chilled 50 mm Hepes (pH 7.4), supplemented with protease and phosphatase inhibitors, was added to each culture dish, the cellular material was scraped off the dish, and the resulting cell lysate was syringed several times through a needle. These total homogenates mixed with 0.85M sucrose solution in a total volume of 2 mL. The homogenates were transferred to a centrifuge tube, gently over layered with 1 mL of 0.25 m sucrose, and centrifuged at 75000 ***g*** for 1.5 h at 4°C using a Beckman Coulter MLA-55 rotor. The lipid-rich, myelin fraction was visible at the gradient of 0.85/0.25 m sucrose. It was harvested and transferred to a 2-mL tube. The myelin fraction was subjected to two rounds of hypotonic shock by the addition of five volumes of chilled distilled H_2O, and the myelin extract was pelleted by centrifugation for 30 min at 13000 ***g***. The final pellet was resuspended in 50 mm Hepes supplemented with protease and phosphatase inhibitors, and the protein concentrations of the total homogenate of myelin fraction were determined using the Qubit 2.0 Flurometer.

2.14: Cell Treatments:

2.14.1: Neurotrophins treatment

To Neural stem cells, NSCs were trypsinized and seeded on animal tissue treated cell culture plates for adhesion for overnight time period. Next day cells were incubated with NGF (10 and 50 ng/mL, Invitrogen) and BDNF (10 and 50 ng/mL, Invitrogen) for 10 days for differentiation assay. Every three days media was replaced by freshly prepared treatment medium. In case of Cell survival and proliferation assay same procedure was followed, just NGF and BDNF treatments' time period was 24 hours.

In Oligodendrocytes cells, to study the role of neurotrophins in oligodendrocytes cells lineage, firstly OPC were isolated from mix glial cells and seeded on PLL coated culture dishes. Next day of the seeding cells were ready for treatment and NGF (10ng/mL) and BDNF (10ng/mL) were exposed to cells for 24 hrs to study the proliferation assay. For migration assay, treatment was given for 24, 48 and 72 hours. 5 days continious exposure was given for differentiation of OPC to mature oligodendrocytes.

For Myelinating spinal cord cells, to study the remyelination potential of neurotrophins, firstly culture was treated with TNF-α (1, 10 and 20ng/mL) in dose depended manner. 20ng/mL TNF-α dose was identified as effective dose for demyelination in myelinating spinal cord culture. After confirmation with this TNF-α dose, cells were treated with TNF-α (20ng/mL) alone and TNF-α (20ng/mL) + Neurotrophins (20ng/mL) group to study remyelination.

2.14.2: 17β-estradiol treatment

Study the role of hormonal (17β-estradiol) activated Astrocytes in regulation of CNS myelination. Astrocytes were isolated from mixed glial cell culture and seeded on PLL coated culture dish for experiments. 17β-estradiol (100nM, Himedia) was added, with 1% antibiotic contain serum free medium, to cells for 6, 12 and 24 hours to study its direct and indirect action on neurotrophins expression which are very essential for regulation of CNS myelination. RNA and whole protein lysate were collected from treated group and performed PCR and western blot.

2.14.3: Inhibitor treatment

For the inhibition studies of membrane receptors and signalling pathways, following inhibitors/blockers were used (Table 4). Concentration and exposure time were determined using previous studies.

Neurotrophins receptors inhibitors study

Cells were grown as described. Cells were seeded on Poly-L-lysine (PLL) treated well plate. Initially Cells were treated with TrkA inhibitor and TrkB inhibitor for 1 hour followed by NGF (10ng/mL) and BDNF (10 ng/mL) treatment for proliferation, migration and differentiation for NSC and OPCs. Cells were fed by renewing the same treatment every three days till the end of the experiments.

Estradiol receptor and ERK signaling inhibitor

Isolated astrocytes were plated in DMEM containing 10% FBS and 1% penicillin/streptomycin on PLL coated culture plates. After reaching to confluency (1 x 10^6 cells) astrocytes were pre-incubated with ERα antagonist, ERβ antagonist and 25μM MEK inhibitor for 1 hour followed by treatment with 100nM 17β-Estradiol (1, 3, 5-Estratreine-3,17β-diol; HiMedia) (Lorenzo *et al.*, 1992, Lee & McEwen, 2001, Kajta *et al.*, 2006) for three different time points- 6hrs, 12hrs and 24hrs. The time course for the analysis was decided to follow both the genomic and non-genomic action of estradiol (Lee & McEwen, 2001).

Table 4: Antagonists and their concentration for Cell treatments

Antagonist/ Inhibitor	Concentration	Function	Company	References
GW 441756	1μM	TrkA inhibitor	Tocris	(Lawn *et al.*, 2015)(Wang *et al.*, 2008)(Zhang *et al.*, 2014)
ANA12-CAS	10μM	TrkB inhibitor	Tocris	(Cazorla *et al.*,

				2011)
MPP Dihydrochloride	10μM	ERα antagonist	Sigma	(Bains *et al.*, 2007, Dong *et al.*, 2009)(Dong *et al.*, 2009)
4-Hydroxytamoxifen	10μM	ERβ antagonist	Sigma	
PD098059	25μM	MEK inhibitor for ERK signaling	Invitrogen	(Blázquez *et al.*, 2000, Choi *et al.*, 2011)

2.15: 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 analyses were carried out using Prism 6 software (GraphPad Software Inc.). A value of $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).