Chapter 3
To determine the role of BDNF, NGF and their receptors in the regulation of Neural Stem Cell differentiation into Oligodendrocytes

3.1. Introduction:

Neural stem cells (NSCs) are dynamic population of cells that contribute new neurons, astrocytes and oligodendrocytes to the brain throughout life and have been shown to have potential applications in cell replacement therapy (Bithell & Williams, 2005, Tang *et al.*, 2017). NSCs are involved in both neurogenesis and gliogenesis. The differentiation of NSCs in astrocytes, oligodendrocytes and neurons is very crucial for disease affected brain such as MS, Alzheimer, Devic's disease and Leukodystrophies to alleviate the normal condition. Proliferation and differentiation of NSCs are not only regulated by the endogenous genes (Artavanis-Tsakonas *et al.*, 1999, Osawa *et al.*, 2005), but are also closely related with various factors including neurotrophic factors in the local microenvironment (Benoit *et al.*, 2001). Existing neurotrophic factorsin the local microenvironment of the brain play an important role in protecting neural functions and repairing brain injuries by supporting the survival of neurons, promoting their growth and differentiation, and also maintaining their functions (Joannides *et al.*, 2007, Wang *et al.*, 2006). Moreover, the effects of BDNF and NGF have been reported to exert a marked impact on the proliferation and differentiation of NSCs (Liu *et al.*, 2014, Chen *et al.*, 2014). Involvement of neurotrophins in neural stem cell differentiation has been a major focus since last one decade. However, very little is known in regards to the modulation of cell fate decision of endogenous NSCs and OPCs via appropriate signaling mechanism involving these neurotrophins' action in producing large number of oligodendrocytes and enhancement of remyelination which is a hallmark feature of many demyelinating diseases.

The present study aims to provide the contribution of exogenous neurotrophins in mediating NSC differentiation to oligodendrocytes, and the role of these neurotrophins receptors (TrkA & TrkB) in presence of their specific pharmacological inhibiters. Thus, this study aims to provide valuable information behind the mechanism governing NSCs differentiation and involved neurotrophins.

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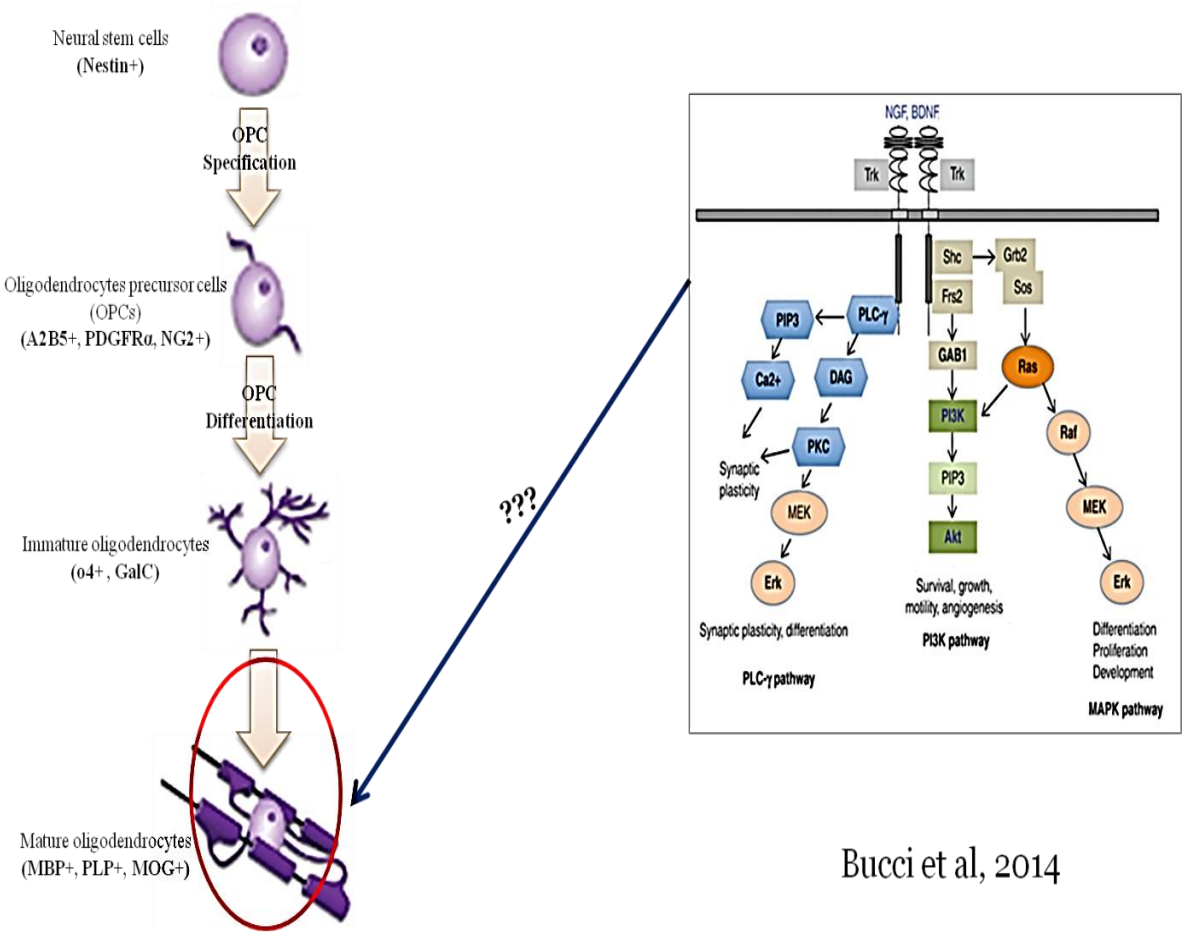

Bucci et al, 2014

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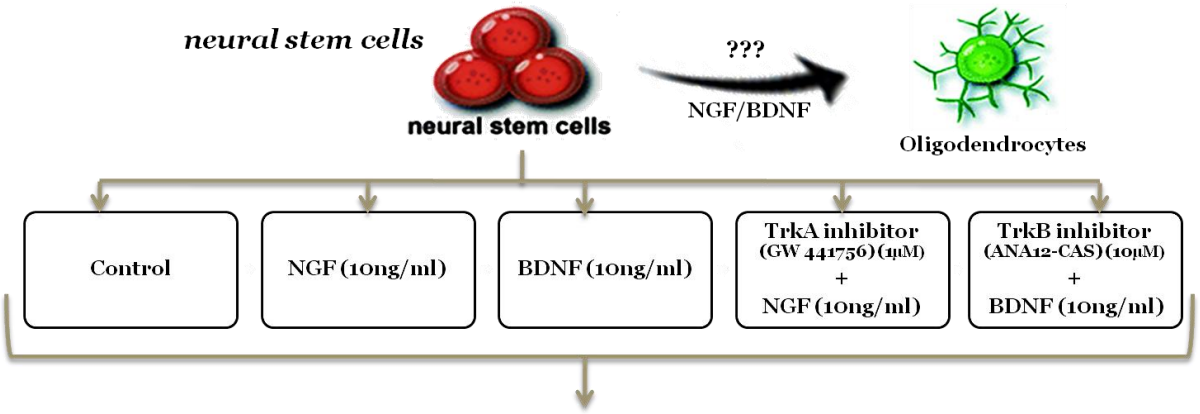
3.2. Strategy of work:



- **After 10 days of treatment** *(Ahmed et al.1995, Liu et al. 2014)*: Differentiation into mature cells was analysed by western blot and real time PCR of **Tuj-1 (neuron), MBP (oligodendrocytes), GFAP (Astrocytes).**
- Neurotrophins' signalling mechanism was investigated by immunoblotting of activated **ERK (p-ERK).**

3.3 Results:

3.3.1: Isolation and Characterization of Neural stem cells

Neurosphere culture was established from the brain cortex of charles foster rat pups (n=10-12) post natal day (P0-P1) (Laura Pacey *et al.*, 2006). Brain cortices were digested in serum free DMEM:F12 media containing 20ng/ml EGF, 20ng/ml FGF, 0.22µM Insulin and 1X B27 supplement to generate neurospheres. Dissociated cells were plated in non-treated tissue culture flasks. Cells were maintained at 37°C in an incubator with 5% CO_2. Single cells began to form spheres within 5 to 7 days of suspension culture and continued to grow in mass and number during the next few weeks. Half of the medium

was changed every 4-5 days. Neurospheres from passage numbers 2 to 3 were used for the experiments. Cells were characterized by immunocytochemistry using cell specific markers (Fig. 3.1)

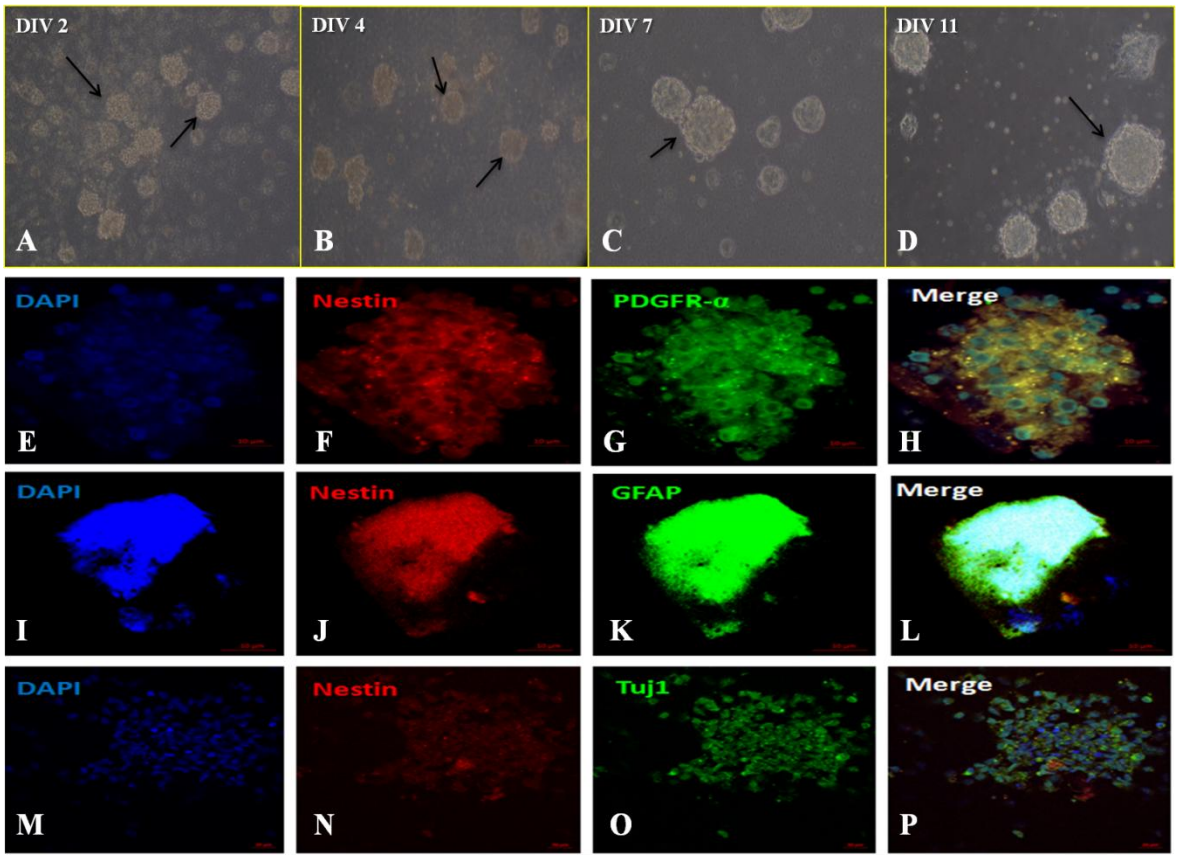


Figure 3.1: Neurosphere Culturing and Characterization of Neural stem cells isolated from P0 rat pups: *Sphere culture from cortical region of P0-P1 day neonatal rat pups. (A-D)Phase contrast images of neural stem cell culture (day wise). Arrows indicate sphere of progenitor cells.DIV 9 cells were used for immunofluorescent staining with specific cell marker antibodies. (E-H) Co-localization of Nestin and PDGFR along with DAPI. (I-L) GFAP and Nestin protein co-localization in DIV 9 NSC. (M-P) Co-localization of Tuj1 and Nestin.All images were takenat 63X inImmunofluorescent microscope using Anti-Nestin (Red), Anti-PDGFR-α (Green), Anti-GFAP (Green), Anti-*

Tuj 1 (Green) monoclonal antibodies visualized with corresponding FITC and TRITC secondary antibodies; DAPI stain for nucleus identification. Scale Bar= 10µm

3.3.2: Role of BDNF & NGF in neural stem cell survival

Cell viability of NSCs was assessed by MTT assay to determine the cytotoxicity of neurotrophins. NSCs were treated with different doses of NGF and BDNF (10 and 50 ng/mL) for 24hrs followed by the MTT assay (Li *et al.*, 2017). Data revealed that exogenous NGF and BDNF did notaffectthe cell viability of NSCswith both doses. The percentage survival of NSCs was significantly increased upon incubation with 10 and 50 ng/mL of NGF (Fig.3.2A) and 50 ng/mL BDNF compared with the individual untreated control (Fig.3.2B).

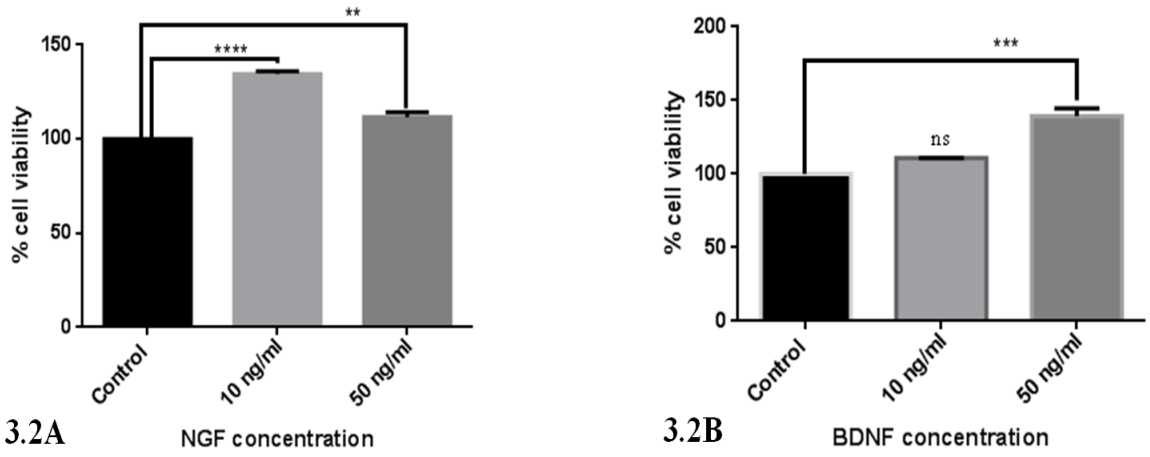
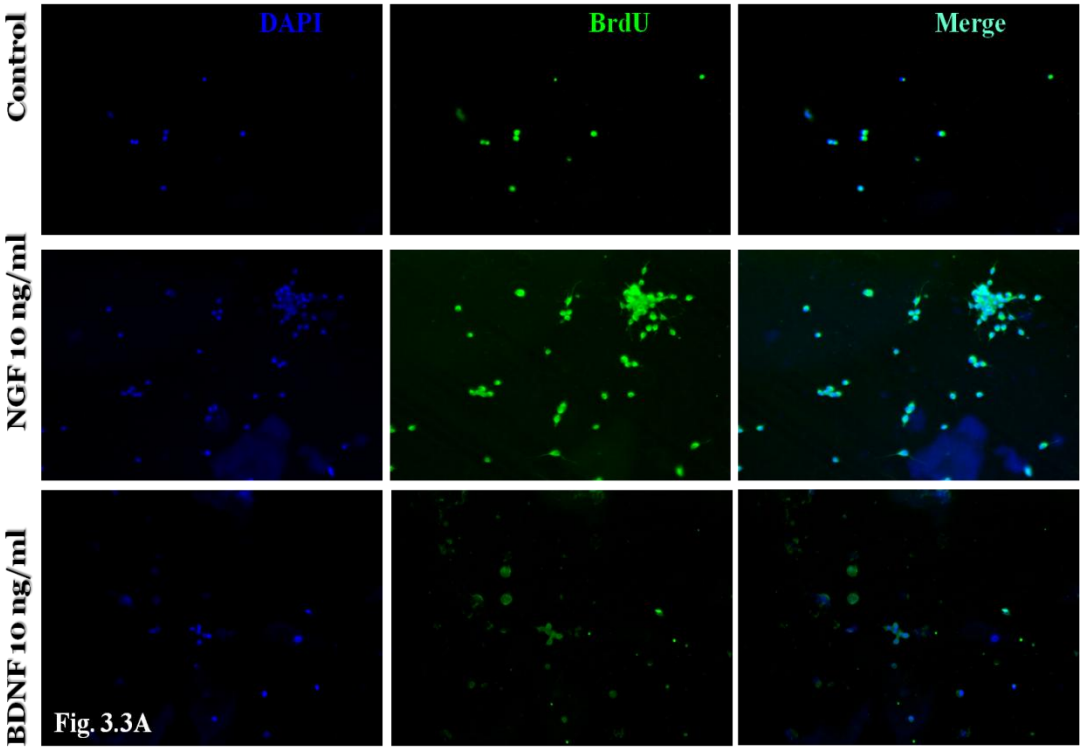


Figure 3.2: Role of BDNF & NGF in neural stem cell survival: *NSCs were treated with 10ng/ml and 50ng/ml of NGF and BDNF followed by measuring percentage cell viability by MTT assay with respect to corresponding control. Data of three independent experiments and treatment in duplicate are mentioned. These data were analysed by one-way ANOVA followed by Bonferroni test and presented as mean ± SEM of 3 replicate measurements (*P<0·05, **p < 0.01, ***P< 0.001) compared with control.*

3.3.3: Role of BDNF & NGF in neural stem cell proliferation

Passage 3 neurosphere cell suspensions were centrifuged, cell pellet was isolated and the supernatant wasdiscarded. Cells were seeded on poly-L-lysine-coated coverslips in 6-well plates and supplemented with culture media containing 10% fetal bovine serum.Cells were treated with 50 mg/L 5-bromo-2′-deoxyuridine (BrdU) (Boster, Wuhan, China) and cultured in a saturated humid incubator at 37°C with 5% CO_2 for 6 hours. Cells mounted on coverslips were fixed for 10 minutes using cold acetone, and nestin (Chemicon, Billerica, MA, USA) and BrdU (Accurate Chemical, Westbury, NY, USA) double immunofluorescence staining performed. The remaining slides were cultured for 7 days in medium containing serum, followed by fixation and prepared for immunofluorescence staining using microtubule associated protein-2 (MAP2) and glial fibrillary acidic protein (GFAP) (Chemicon). Slides were observedby inverted fluorescence microscopy.

BrdU assay was performed to evaluate the proliferation of NSCs in presence of neurotrophins. NSCs were seeded on PLL coated coverslips and treated with 10ng/ml NGF and BDNF (Soltys*et al.*,2011). After 24 hours BrdU assay was performed as described in materials and methods chapter (Chapter-2). The ratio of BrdU-positive cells vs DAPI-positive cells (total cell number) was evaluated to obtain the number of proliferating cells. Result shows that NGF promotes NSC proliferation*in-vitro* compared to control while BDNF is not involved in proliferation process of the NSCs (Fig.3.3).



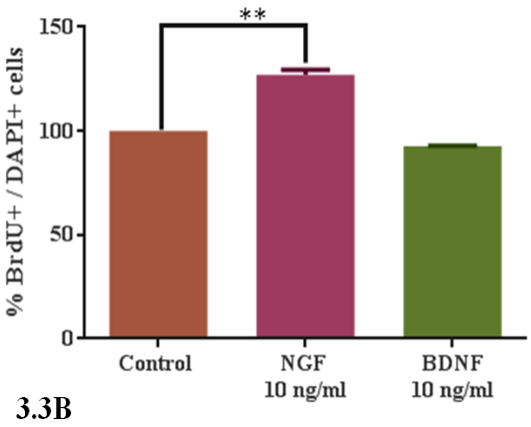
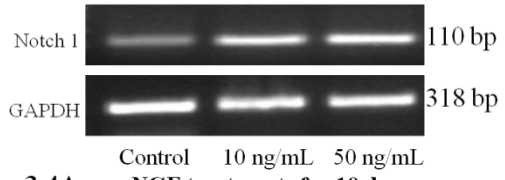


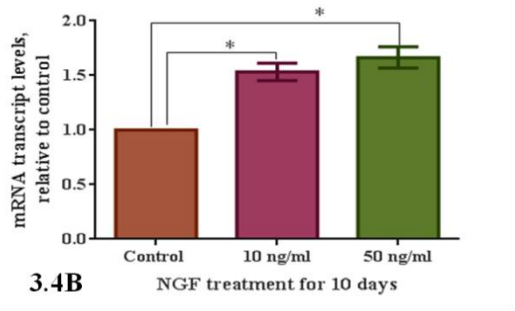
Figure 3.3: Role of BDNF & NGF in neural stem cell proliferation: *(3.3A) Double immunofluorescence staining of BrdU and DAPI in NGF and BDNF treated NSCs. (3.3B) BrdU - positive cells were divided by DAPI-positive cells (total cell number) to obtain a ratio of proliferating cells. Results were evaluated by one-way ANOVA and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

3.3.4:Validation of gliogenesis by evaluation of Notch-1 expression

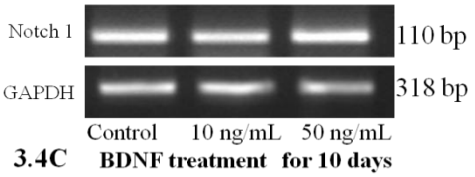
Notch ligands are marked candidates for neuro-glial switching (Morrison *et al.*, 2000). Notch signaling has been shown to inhibit neuronal differentiation in both invertebrate and vertebrate systems (Coffman *et al.*, 1993, Artavanis-Tsakonas *et al.*, 1999, Henrique *et al.*, 1997, Zhou *et al.*, 2010). Notch family genes are expressed by neural stem cells (Weinmaster *et al.*, 1991, Williams *et al.*, 1995). Notch-1 expression was assayed to evaluate whether or not NGF and BDNF promotes gliogenesis. NSCs were seeded on PLL coated culture plate followed by NGF and BDNF treatment (10 and 50 ng/mL) for 10 days. Treatment medium was replaced every third day. After 10 days, RNA was isolated from cell lysate using Trizol reagent (Invitrogen)and Notch-1 gene expression was assessed by semi quantitative PCR. Results indicate expression of Notch-1 was up-regulated in dose dependent manner in NGF while only 50ng/ml dose of BDNF resulted in up-regulated levels of Notch. Thus, both neurotrophins trigger gliogenesis in NSCs differentiation (Fig.3.4).



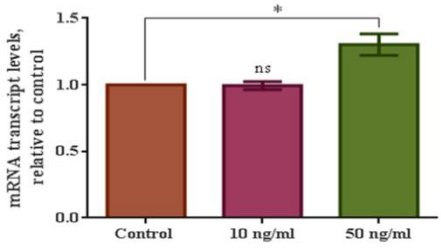
3.4A NGF treatment for 10 days



3.4B NGF treatment for 10 days



3.4C BDNF treatment for 10 days



3.4D BDNF treatment for 10 days

Figure 3.4: Notch expression study to confirm triggering of gliogenesis: *NSCs were treated with NGF and BDNF after which Notch transcript levels were detected by semi quantitative PCR. 3.4A and 3.4C are typical pattern of gene expression of Notch following NGF and BDNF treatment while 3.4B and 3.4D are graphical representation of mRNA expression, normalized to GAPDH and relative to control. Data of three independent experiments were analysed by One-way ANOVA followed by Bonferroni test and presented as mean ± SEM of 3 replicate measurements (*P<0·05, **p < 0.01, ***P< 0.001) compared with control*

3.3.5: NGF and BDNF mediated regulation of NSC differentiation in oligodendrocytes

Phase contrast morphological observations suggest NSC differentiation is regulated by NGF and BDNF, which is evident by increased cell outgrowth from neurospheres in treated cells compared to control (Fig.3.5 (a)A) and 3.5 (b)A). NSCs were cultured in differentiation medium consisting of DMEM/F12 (1:1) supplemented with 2% B27 supplement, 1% FBS along with NGF/BDNF treatment (10ng/ml & 50 ng/ml) for 10 days followed by transcript (Fig. 3.5(a)D) and 3.5(b)D) and protein analysis (Fig. 3.5(a)C) and 3.5(b)C) of Myelin Basic Protein (MBP; mature oligodendrocyte marker). We found significant up-regulation in transcript and protein levels of MBP when treated with both NGF and BDNF in a dose dependent manner. Further, we observed increased expression of GFAP (astrocyte marker) and no change in Tuj 1 (neuronal marker) expression in neurotrophin treatment groups compared to control. These observations suggest that neurotrophins, especially higher doses favor gliogenesis more than neurogenesis.

NGF regulated oligodendrocytes differentiation from NSCs:

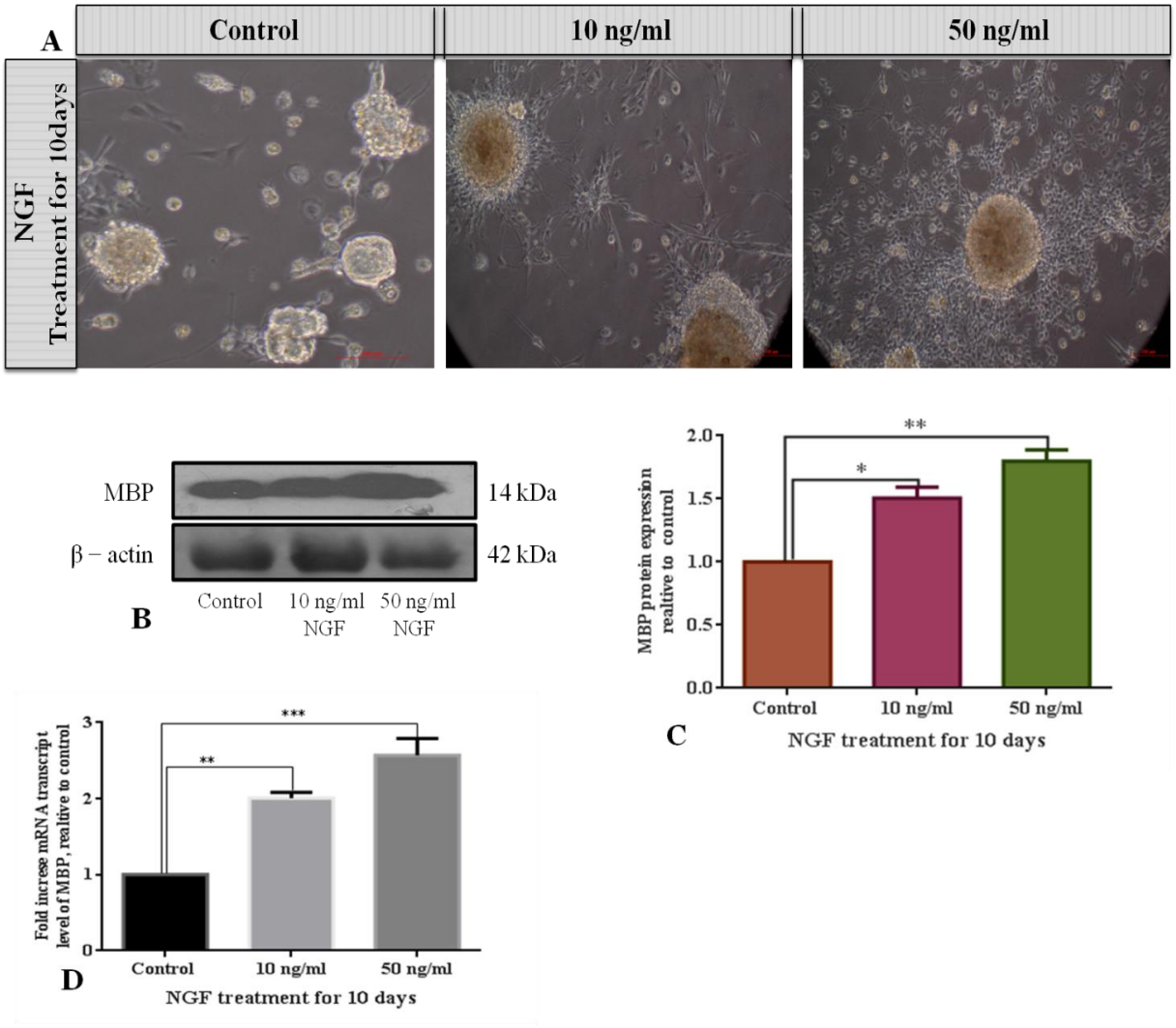


Fig.3.5 (a): NGF regulates Oligodendrocyte differentiation from NSCs: *NSCs were treated with 10ng/mL and 50ng/mL NGF. A) Phase contrast observations of NSC differentiation. B) Representative western blot image of MBP protein level. C) Graphical representation of MBP protein levels, normalized to β-actin and relative to control. D) Graphical representation of mRNA expression, normalized to GAPDH and relative to control. Results were evaluated by one-way ANOVA and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

BDNF regulated oligodendrocytes differentiation from NSCs:

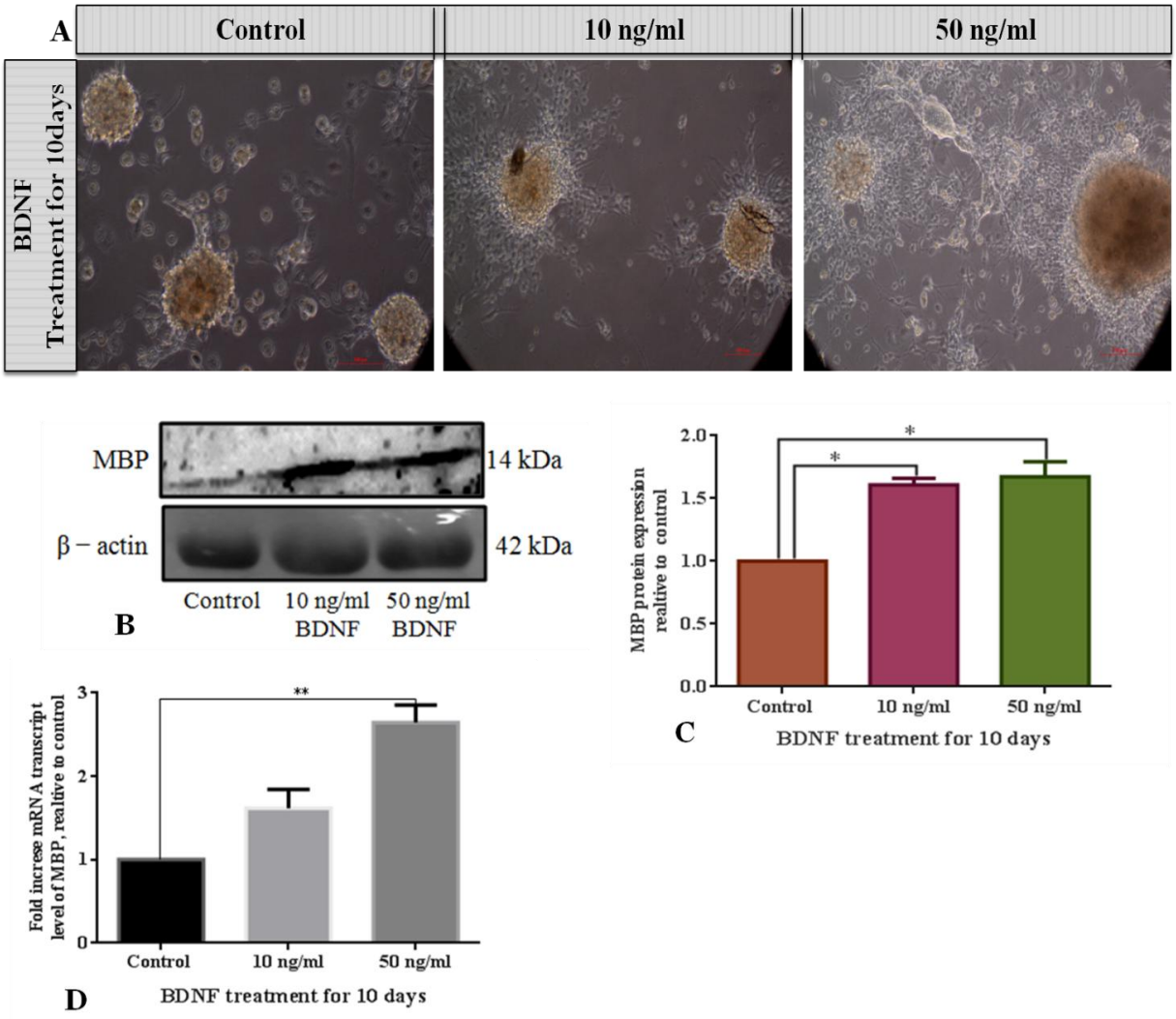
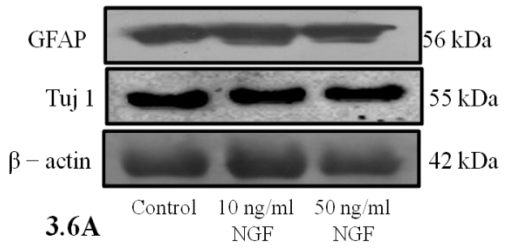
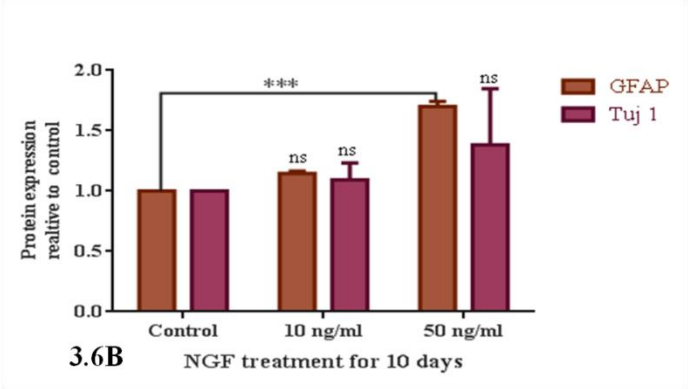


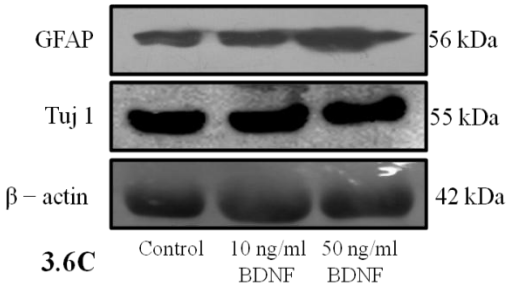
Fig. 3.5 (b): BDNF regulates Oligodendrocyte differentiation from NSCs: *NSCs were treated with 10ng/mL and 50ng/mL BDNF. A) Phase contrast observations of NSC differentiation. B) Representative western blot image of MBP protein level. C) Graphical representation of MBP protein levels, normalized to β-actin and relative to control. D) Graphical representation of mRNA expression, normalized to GAPDH and relative to control. Results were evaluated by one-way ANOVA and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

3.3.6: Differentiation of NSCs to Astrocytes and Neuron with treatment of NGF & BDNF

NSCs were cultured in differentiation medium for 10 days followed by protein analysis of GFAP and Tuj1; a marker for Astrocytes and Neurons respectively. We found that in both neurotrophins' treatment at higher dose (50 ng/mL) the expression of GFAP was up-regulated while Tuj1 expression was unaltered which suggests that both NGF and BDNF, favor gliogenesis over neurogenesis, especially at higher doses In contrast there was no alteration in the GFAP andTuj1 expression at lower dose (10 ng/mL) of NGF and BDNF (Fig. 3.6).







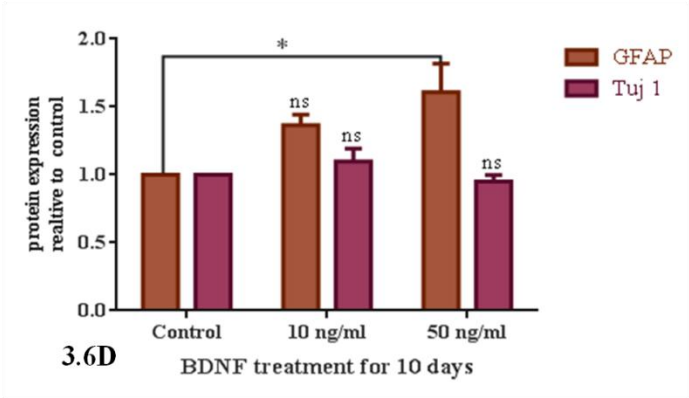


Fig. 3.6: NGF and BDNF regulate Astrocytes and neuron cells differentiation from NSCs: *NSCs were treated with 10ng/mL and 50ng/mL of NGF and BDNF. A) Representative western blot image of GFAP and Tuj1 proteins level of differentiated NSCs*

*treated with NGF. B) Graphical representation of proteins levels, normalized to β-actin and relative to control. C) Representative western blot image of GFAP and Tuj1 proteins level of differentiated NSCs treated with BDNF. D) Graphical representation of proteins levels, normalized to β-actin and relative to control.Results were evaluated by one-way ANOVA and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

3.3.7: Involvement of TrkA&TrkB in Oligodendrocyte generation through ERK pathway

NGF and BDNF act through their specific receptors- TrkA and TrkB respectively. To confirm the role of this specific action in oligodendrogenesis, TrkA and TrkB receptors were blocked for 1hr using pharmacological specific inhibitors (Lawn *et al.*, 2015, Wang *et al.*, 2008, Zhang *et al.*, 2014, Cazorla *et al.*, 2011)followed by exogenously providing NGF (10ng/mL) and BDNF (10ng/mL) respectively for 10 days. The treatment, with and without inhibitors, was repeated every 3 days. Myelin protein, MBP expression level, was analyzed at the end of treatment. Significant down-regulation of MBP expression was observed in inhibitor group compared to only neurotrophins treated groups (Fig. 3.7(a)) which indicates specific role of Trk receptors (TrkA and TrkB) in neurotrophins mediated NSC differentiation into oligodendrocytes. ERK has been well studied as a down-stream signaling pathway of NGF and BDNF where it plays an important role in cell differentiation, proliferation and development whereas Akt pathway has been implicated in cell survival, growth and motility (Arevalo & Wu, 2006). This encouraged us to study the role of ERK specifically in NSC differentiation in response to neurotrophins. Phosphorylated ERK levels were significantly down-regulated in Trk inhibitor groups compared to only neurotrophin treated groups (Fig. 3.7(b)) which suggests that the regulation of decreased MBP levels by Trk inhibitors is via the ERK pathway.

Involvement of TrkA & TrkB in oligodendrocytes differentiation:

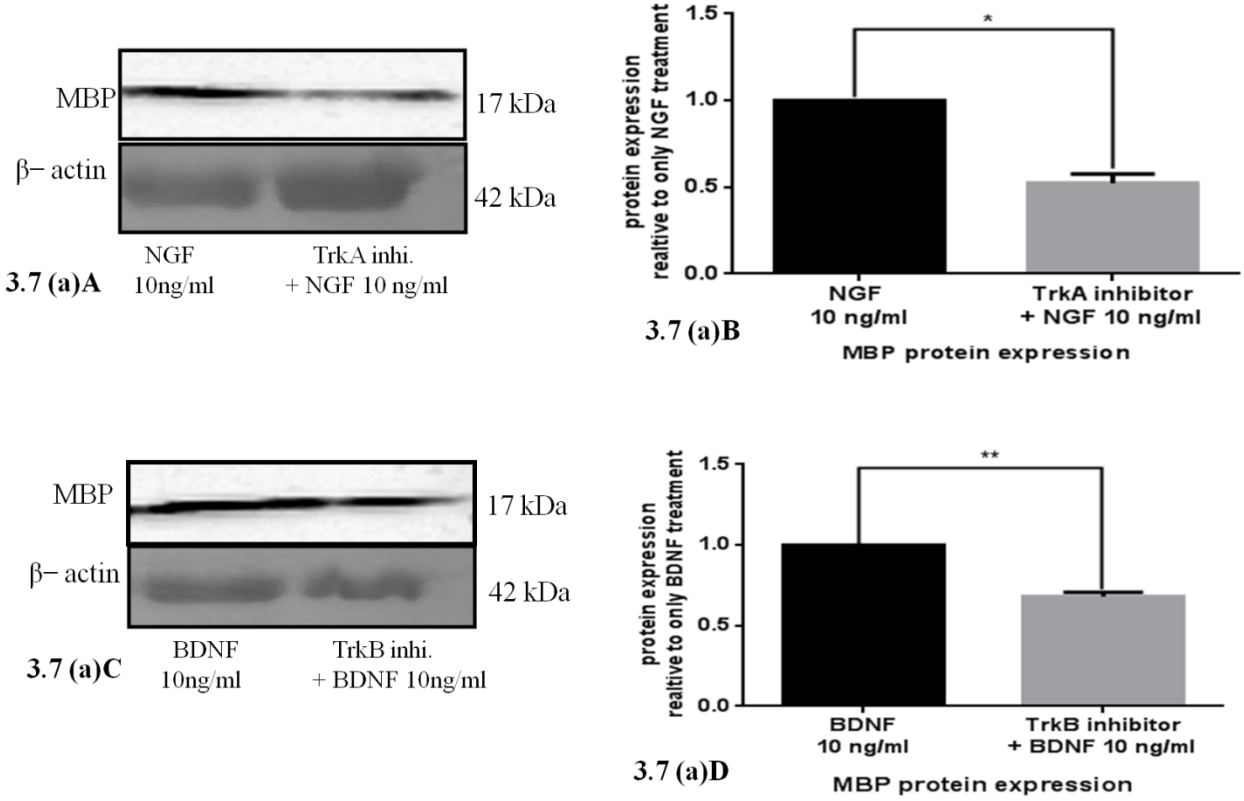
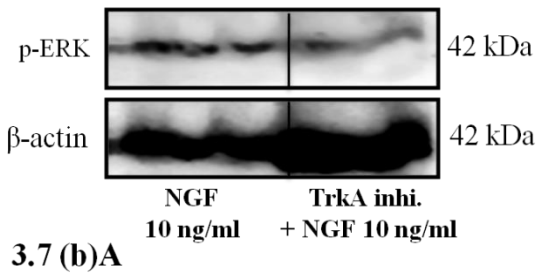
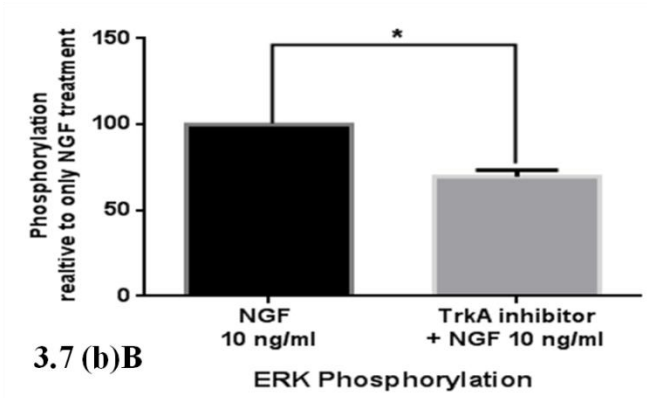


Figure 3.7(a): Involvement of TrkA&TrkB in Oligodendrocyte differentiation: *NSCs were treated with Trk receptor inhibitors for one hour followed by treatment with 10ng/ml concentration of NGF and BDNF. A and C) Representative western blot image of MBP protein level. B and D) Graphical representation of MBP protein levels, normalized to β-actin and relative to control. Results were evaluated by student's t-test and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

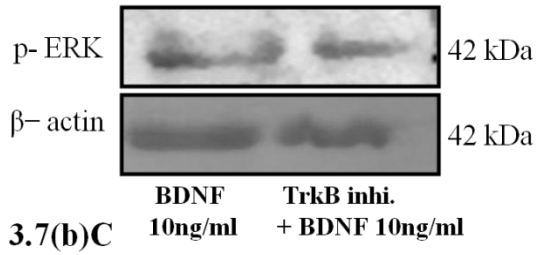
Involvement of TrkA&TrkB in Oligodendrocyte generation through ERK pathway:



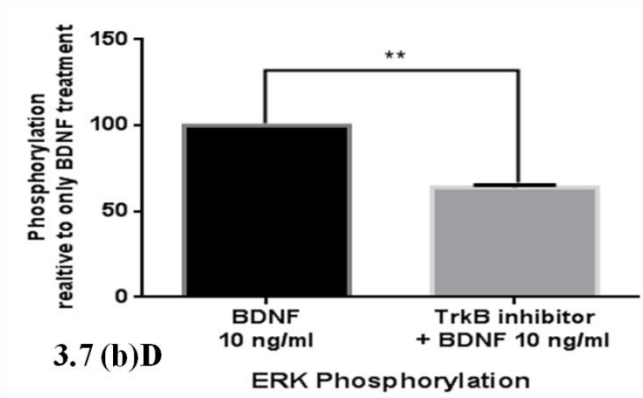
3.7 (b)A



3.7 (b)B



3.7(b)C



3.7 (b)D

Figure 3.7(b): Involvement of TrkA&TrkB in Oligodendrocyte generation through ERK pathway: *NSCs were treated with Trk receptor inhibitors for one hour followed by treatment with 10ng/mL concentration of NGF and BDNF. A)And C) Representative western blot image of p-ERK protein level. B and D) Graphical representation of p-ERK protein levels, normalized to β-actin and relative to control. Results were evaluated by student's t-test and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

3.3.8: Morphological analysis of Neurotrophins (NGF & BDNF) treated NSCs

Morphometery analysis of NGF and BDNF treated NSCswas employed to study the morphology modulation. Cells were treated with NGF and BDNF along with or without receptor inhibitor. Post treatment numbers of neurosphere were counted by phase contrast microscope. Number of colonies was counted after treatment of NGF and BDNF for 72 hours which showed that NGF increased the number of neurospheres. On the other hand TrkA receptor (NGF specific receptor) inhibitor treatment significantly down regulated numbers of neurospheres even in the presence of NGF (Fig. 3.8A) which indicated NGF promotes proliferation of NSCs. In the case of BDNF, neurosphere count was not altered which suggest neutral role of BDNF (Fig. 3.8B) in NSCs proliferation.

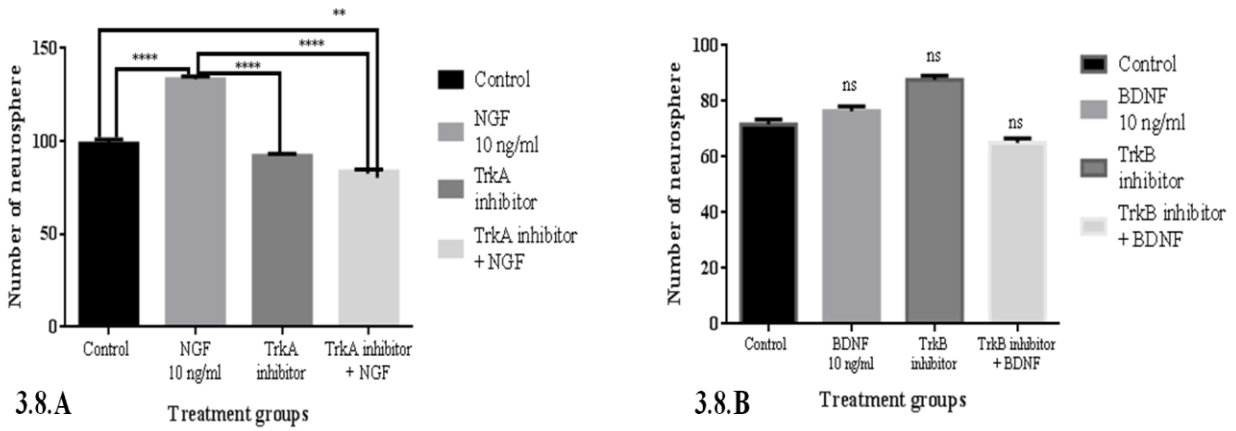
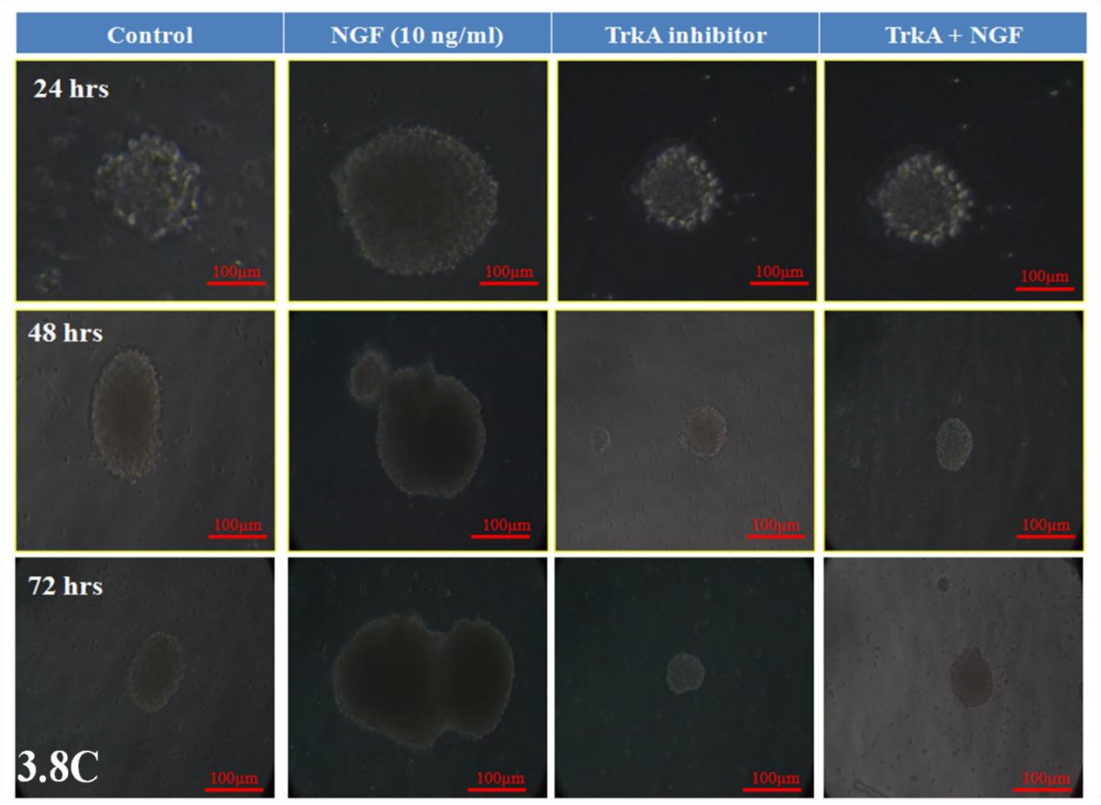


Figure 3.8A & 3.8B: Numbers of colony after treatment of NGF and BDNF: *NSCs were treated with Trk receptor inhibitors for one hour followed by treatment with 10ng/mL concentration of NGF and BDNF for 72 hours. 3.8.A) Graphical representation of Neurosphere count after NGF treatment with and without TrkA inhibitor treatment compare with control. 3.8.B) Graphical representation of Neurosphere count after BDNF treatment with and without TrkB inhibitor treatment compare with control. Results were evaluated by one-way ANOVA and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

NGF mediated modulation of neurosphere:

NSCs were treated with following group of treatment: Control, NGF alone, TrkA inhibitor alone, TrkA inhibitor + NGF and morphological analysis was done through phase contrast microscopy (Fig. 3.8C). Size of neurosphere was measured using *NIS software* (Elements microscope imaging software) at three different time points: 24, 48 and 72 hours. Results suggested that NGF significantly increase the size of neurosphere in all time point compare to control (Fig. 3.8C, D).On the other hand TrkA receptor blocker reduced the size in addition after NGF treatment, also TrkA inhibitor was not able to increase the size of NSCs' colony (Fig.3.8C, D) which clearly suggests the action of NGF in modulation of neurosphere assay through TrkA receptor only.



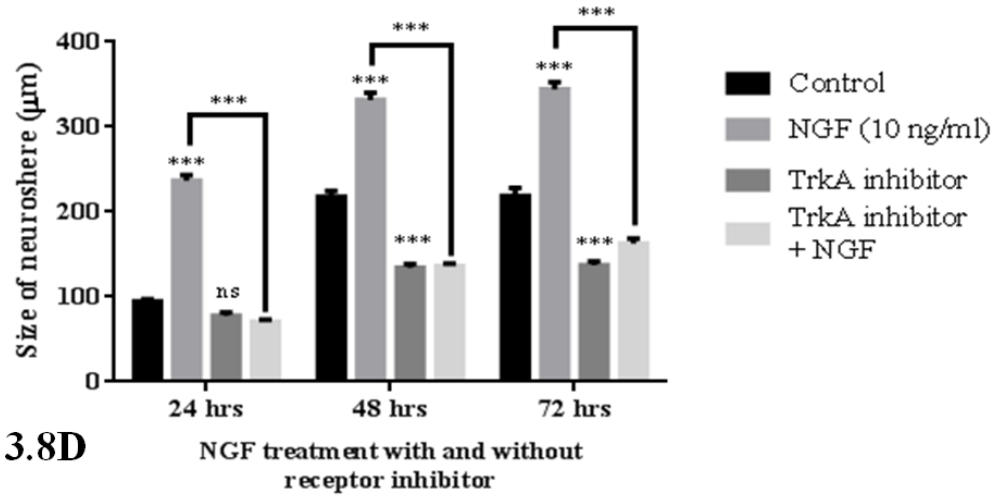
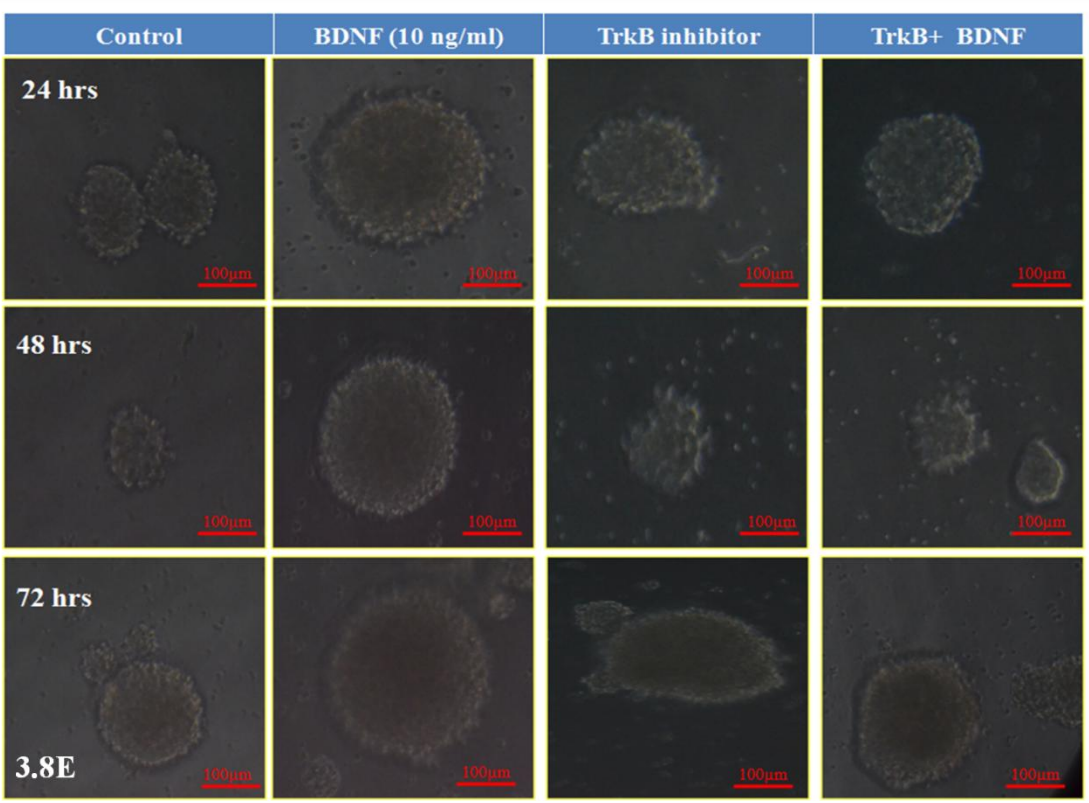


Figure 3.8C & 3.8D: Morphological analysis of Neurosphere size assay with NGF treatment: *NSCs were treated with Trk receptor inhibitors for one hour followed by treatment with 10ng/mL concentration of NGF. Neurosphere size was measured at 24, 48 and 72 hrs time point. 3.8C) Phase contras imaging of modulated size of Neurosphere in NGF treatment, with and without TrkA inhibition, compare to control. 3.8D) Graphical representation of size of neurosphere (µm) compare to control. Results were evaluated by one-way ANOVA and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

BDNF mediated modulation of neurosphere:

NSCs were treated with following group of treatment: Control, only BDNF, only TrkB inhibitor, TrkB inhibitor + BDNF and morphological analysis was done through phase contrast microscopy (Fig. 3.8E) Size of neurosphere was measured using NIS software (Elements microscope imaging software) at three different time points: 24, 48 and 72 hours. Results suggested BDNF significantly increase the size of neurosphere in all time point compared to control (Fig. 3.8E, F) while BDNF treatment along with TrkB receptor blocker reduced the size (Fig.3.8E, F) which clearly suggests the role of BDNF in neurosphere modulation through its TrkB receptor.



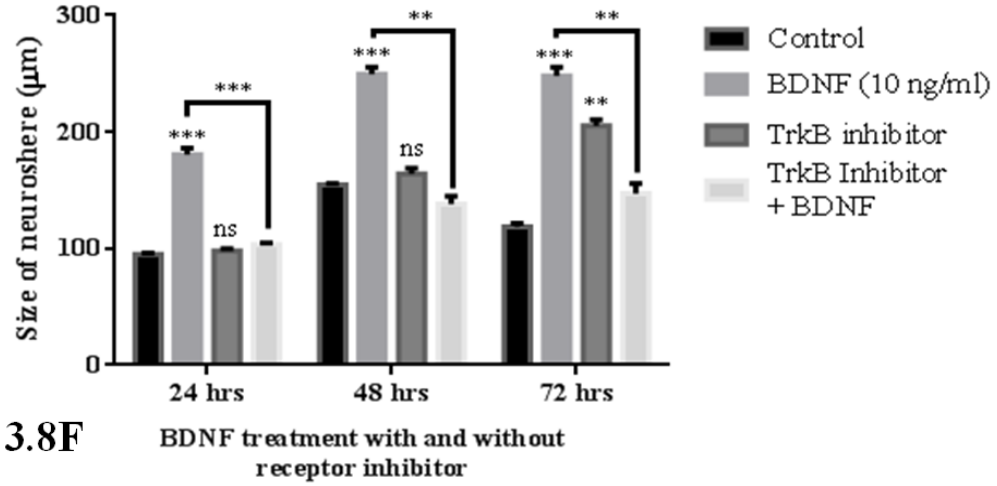


Figure 3.8E & 3.8F: Morphological analysis of Neurosphere size assay with BDNF treatment: *NSCs were treated with Trk receptor inhibitors for one hour followed by*

*treatment with 10ng/mL concentration of BDNF. Neurosphere size was measured at 24, 48 and 72 hrs time point. 3.8E) Phase contrast imaging of modulated size of Neurosphere in NGF treatment, with and without TrkB inhibition, compare to control. 3.8F) Graphical representation of size of neurosphere (µm) compare to control. Results were evaluated by one-way ANOVA and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

3.4: Discussion

Neural stem cell culture was established and the stem cell nature was confirmed by the presence of Nestin by immunocytochemical analysis. These neurospheres were also positive for Tuj1, GFAP and PDGFR-α- cell specific markers of neurons, astrocytes and oligodendrocytes precursor cells respectively. Neurotrophins are a class of growth factors promoting proliferation and differentiation of neural stem cells (Liu *et al.*, 2014, Chen *et al.*, 2014). These NSCs have also been reported to express neurotrophin receptors (Young *et al.*, 2007). However, there are no reports on the signaling mechanism behind the action of neurotrophins. In the present study, we have shownthe involvement of NGF in the survival and proliferation of NSCs where 10ng/ml was the effective dose. On the other hand, BDNF regulates only cell viability while exhibiting no effect on the proliferation capacity of NSCs. Thus, both the exogenous recombinant neurotrophins (NGF and BDNF) does not cause cell death. Both neurotrophins significantly lead to the up-regulation of Notch, a key molecule for neuron-glial switching (Morrison *et al.*, 2000). Notch is known to inhibit neuronal differentiation (Artavanis-Tsakonas *et al.*, 1999), thus elevated levels of this molecule by NGF and BDNF suggests potential role of neurotrophins in driving differentiation towards gliogenesis lineage. Our findings show up-regulation of MBP in both transcript and protein levels in dose dependent manner in neurotrophins treated groups compared to control. These observations suggest NGF and BDNF mediated activation of oligodendrocyte specific lineage from NSCs. To confirm the role of NGF and BDNF in NSC differentiation, pharmacological inhibitors studies

were carried out using specific inhibitors for neurotrophin receptors- TrkA and TrkB for NGF and BDNF respectively. Inhibition of Trk receptors resulted in significant down-regulation of MBP expression compared to without inhibitor groups which suggests the action of NGF and BDNF in oligodendrocyte differentiation through Trk receptors. ERK is one of the main downstream signaling pathway for NGF and BDNF, majorly involved in cell differentiation and proliferation (Arevalo & Wu, 2006). Phosphorylated ERK (p-ERK) levels were down-regulated in TrkA and TrkB inhibited cells compared to without inhibitor groups suggesting that ERK act as a major neurotrophin signaling pathway to direct NSCs into oligodendroglial lineage. Morphometric analysis of neurosphere indicates vital role of neurotrophins in NSCs' size and numbers alteration in upwards manner through both Trk receptors (TrkA and TrkB),clearly suggesting important role of both neurotrophins, NGF and BDNF, in regulation of size and numbers of neurospheres.

In conclusion, our data shows the involvement of NGF and BDNF in differentiation of NSC-derived oligodendrocytes through specific Trk receptors. The study also confirms the involvement of ERK phosphorylation as a major link in the glial cell differentiation particularly into oligodendrocytes. These findings can be elaborated and extrapolated to look at NGF and BDNF as potential therapeutic targets for several demyelinating disorders such as MS, Leukodystrophies and many more. The challenge for further research is to unravel the complex molecular mechanisms such as TrkA and TrkB knock out NSCs differentiation lineage and briefly study about Notch and wnt signaling for understanding of gliogenesis lineage activation and neurogenesis lineage activation. These mechanisms of both neurotrophins' (BDNF & NGF) and their receptors (TrkA & TrkB) will help to understand the vital role of BDNF and NGF in the differentiation and maturation of oligodendrocytes as well as other brain cells.