Chapter 4
To study the signaling mechanism of BDNF and NGF in Oligodendrocyte Progenitor Cells (OPCs) proliferation, migration and differentiation

4.1: Introduction:

Oligodendrocytes are the myelinating cells of the central nervous system (CNS). Mature oligodendrocytes capable of myelination are the end product of a cell lineage as a result of complex, accurate and timed program of proliferation, migration, differentiation and finally myelination (Bradl & Lassmann, 2010). Oligodendrocytes migration is very important for myelination as well as remyelination. Oligodendrocyte precursors (OPCs) originate from neuroepithelial cells of the ventricular zone during embryonic life (Curtis et al., 1988, Hardy & Reynolds, 1991, Hardy & Reynolds, 1993, Pfeiffer et al., 1993). In spinal cord, oligodendrocytes initially arise in ventral regions of the neural tube (Warf et al., 1991). Various growth factors involved in the regulation of OPC migration of which most extensively studied growth factor is PDGF (Frost et al., 2009). Neurotrophins are also involved in migration of other cells; BDNF-a neutrophin, stimulates migration of cerebellar granule cells and promote the migration of cortical neurons (Borghesani et al., 2002) whereas NGF (10ng/ml) enhances migration of Schwann cells in the Peripheral nervous system (Anton et al., 1994). Reports also suggests, presence of neurotrophins receptors (Trk receptors) on oligodendrocytes cells are involved in the most of the essential functions of oligodendrocytes such as oligodendrocytes lineage, survival of immature oligodendrocytes and potentiation of mitogenic effect of other growth factors (Cohen et al., 1996, Grinspan, 2002, Coulibaly et al., 2014). OPC proliferation is also very essential process for regulation of CNS myelination as well as for remyelination. Reports have suggested that many factors are responsible for OPC proliferation (Vela et al., 2002, Levine & Reynolds, 1999, Barres & Raff, 1993). Very few studies have revealed role of BDNF and NGF in OPC proliferation. NGF enhances the survival of differentiated oligodendrocytes (Cohen et al., 1996) whereas BDNF (10 ng/ml) affects its development via TrkB receptor (Van't Veer et al., 2009) and promotes CNS myelination via a direct effect on oligodendrocytes (Xiao et al., 2010). However, the regulatory mechanism by which Trk receptors’ affect OPC proliferation and differentiation is still unclear. Differentiation of Oligodendrocytes is final step towards myelination of axon.
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OPCs differentiation is characterized by a rapid increase in morphological complexity (branching of the cell) followed by expansion of uncompacted myelin membrane (Michalski & Kothary, 2015). Overall, scattered information is available regarding neurotrophins and its Trks receptors in context of oligodendrocytes lineage and this calls for a detailed investigation. Thus, in the present study mechanism governed by both neurotrophins, BDNF and NGF in OPC proliferation, migration and differentiation was studied using reported doses of both the neurotrophins along with specific inhibitors of TrkA and TrkB receptors. Outcome of this study will provided an insight into the role of BDNF and NGF for oligodendrocytes lineage which can be used further as a target for treatment of diseases caused by oligodendrocytes dysfunction.
4.2: Strategy of work:

4.3: Results:

4.3.1: Isolation and characterization of Rat Oligodendrocytes Progenitor cells

Isolation and culturing of Oligodendrocyte Progenitor Cells from rat pups was done by a previously described method (Chen et al., 2007). OPCs were isolated from P0–P2 day rat pup cortices as per the briefly described in Chapter 2, Materials and methods. The isolated OPCs and OLGs were significantly enriched and confirmed using morphological and Immunocytochemical analyses. Results showing phase contrast and confocal microscopic imaging of lineage specific oligodendrocytes cells (Fig. 4.1).
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Figure 4.1: Morphological and Immunocytochemical characterization of oligodendrocytes developmental stages: (A) Oligodendrocytes progenitor cells (OPCs) were plated and allowed to grow in Sato media containing PDGF-A (proliferation medium). hrs post plating, cultures were switched entirely to Sato media without PDGF-A (differentiation medium). Cells were then fixed for 24 hrs after differentiation Day -1 (A and D), Day-5(B and E) and Day-10 (C and F) to evaluate OPCs, immature and mature oligodendrocytes (OLGs) respectively. OPCs were detected by stage specific marker PDGFRα (Red). Similarly O4 (Red) marker was used for immature and MBP (Red) for mature oligodendrocytes. Nuclei were stained (blue) with 4’,6-diamidino-2-phenylindole (DAPI).
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4.3.2: OPC proliferation is regulated via neurotrophins receptors, TrkB and TrkA

To evaluate the precise role of both neurotrophins in OPC proliferation, we have used TrkA receptor specific inhibitor (GW 441756, 1μM) (Lawn et al., 2015, Wang et al., 2008, Zhang et al., 2014) for NGF and TrkB receptor specific inhibitors for BDNF (ANA12-CAS, 10μM) (Cazorla et al., 2011). Experimental setup included the following groups: [1] (a) Control, (b) NGF (10ng/ml), (c) TrkA inhibitor + NGF; [2] (a) Control, (b) BDNF (10ng/ml), (c) TrkB inhibitor + BDNF. After 48 hours, cells were incubated with BrdU solution for 4 h at a final concentration of 3μg/ml followed by overnight incubation with anti-BrdU primary antibody at 4°C and later with FITC conjugated secondary antibody (Fig.4.2a.A and Fig. 4.2b.A). Results indicated that, BDNF significantly up regulated OPC proliferation (Fig. 4.2a.B) while NGF did not affect OPC proliferation. Surprisingly in presence of TrkA inhibitor, proliferation was significantly reduced which suggested that action of NGF in OPC proliferation regulation depend on the activation of TrkA (Fig. 4.2b.B); once TrkA activity goes down, NGF failed to regulate the rate of OPC proliferation in OPC in-vitro system. Similarly, BDNF increased Oligodendrocyte proliferation through TrkB receptor, as TrkB receptor inhibition significantly decreased BDNF mediated OPC proliferation rate. Early reports also suggested that growth factors are responsible for the regulation of OPC proliferation (COLLARINI et al., 1991, Chesik et al., Althaus et al., 1992) however study regarding NGF and BDNF role in this phenomena needs to be clarified. Therefore, in current study we studied OPC proliferation regulation mediated by neurotrophins and the precise role of their receptors in proliferation. Overall, proliferation results indicated that majorly BDNF and Trk receptors (TrkA and TrkB) played a significant role in regulation of OPC proliferation.
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BDNF- TrkB mediated OPC proliferation

![Comparison of OPC proliferation with BDNF and TrkB inhibitor](image)

**A**

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
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**B**

Bar chart showing the percentage of BrdU/DAPI+ cells relative to control.

- **Control**
- **BDNF 10 ng/ml**
- **TrkB inhibitor + BDNF**

### Statistical Significance
- ** ****** for BDNF 10 ng/ml compared to control
- ** ** for TrkB inhibitor + BDNF compared to control
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Figure 4.2a: BDNF – TrkB mediated OPC proliferation by BrdU assay: (A) Representative fluorescence images of BrdU incorporation in OPC upon treatment with BDNF with and without TrkB inhibitor. (B) Graphical representation of % BrdU positive cells in treatment group compared to control. Images were captured from four different fields in each group. Data were analysed by one-way ANOVA followed by Bonferroni test and presented as mean±SEM of three independent experiments (*p<0.05, **p<0.01, ****p<0.0001) compared with control.

NGF- TrkA mediated OPC proliferation
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Figure 4.2b: NGF – TrkA mediated OPC proliferation by BrdU assay: (A) Representative fluorescence images of BrdU incorporation in OPC upon treatment with NGF with and without TrkA inhibitor. (B) Graphical representation of % BrdU positive cells in treatment group compared to control. Images were captured from four different fields in each group. Data were analyzed by one-way ANOVA followed by Bonferroni test and presented as mean±SEM of three independent experiments (*p<0.05, **p<0.01) compared with control.

4.3.3: NGF and BDNF mediated regulation of OPCs migration through TrkA and TrkB receptors

OPC migration is vital process of oligodendrocytes lineage. Here, role of neurotrophins in OPC migration was confirmed by scratch assay (Liang et al., 2007). Experimental setup included following groups; (1a) Control, (b)NGF (10ng/ml), (c) TrkA inhibitor (1μM) + NGF; (2) (a) Control, (b) BDNF (10ng/ml), (c) TrkB inhibitor (10μM) + BDNF. Time period for migration study was 24 h, 48 h and 72 h post treatment. OPCs were seeded on PLL coated cell culture well and wound scratch assay was performed at above mentioned time period for migration study and distance migrated by cells was calculated using NIS software. Results suggested that BDNF regulated OPC’s migration in CNS through TrkB receptor (Fig. 4.3a). BDNF treated OPCs migrated on average 300% in all
time points compared to control while TrkB inhibition significantly prevented OPCs migration which confirms that BDNF regulates OPC migration through TrkB receptor. Similarly, NGF induced ~200% migration compared to control (non-treated group), which was found to decrease on treatment with TrkA inhibitor (Fig. 4.3b). Thus, over all conclusions from these observations is that OPC migration was regulated by both the neurotrophins and their respective receptors (TrkA and TrkB) and they played significant role in this key process of Oligodendrocyte lineage.

**BDNF – TrkB mediated OPC migration**

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Figure 4.3a: Measurement of OPC cell migration mediated by BDNF- TrkB in in-vitro scratch assay: OPCs were grown on PLL coated well plates and treated with BDNF (10ng/ml) with and without TrkB receptor inhibitor. They were then subjected to in vitro scratch assay. (A) Images were captured at 0, 24, 48h and 72h after incubation using phase-contrast microscope. (B) The rate of migration was measured by quantifying the unfilled area of the scratch using NIS software (Elements microscope imaging software). The % migration was represented 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).
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NGF – TrkA mediated OPC migration

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<th>TrkA inhibitor + NGF (10 ng/mL)</th>
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Figure 4.3b: Measurement of OPC cell migration mediated by NGF- TrkA in in-vitro scratch assay: OPCs were grown on PLL coated well plates and treated with NGF (10ng/ml) with and without TrkA receptor inhibitor. They were then subjected to in-vitro scratch assay. (A) Images captured at 0, 24, 48h and 72h after incubation using phase-
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contrast microscope. (B) The rate of migration was measured by quantifying the unfilled area of the scratch using NIS software (Elements microscope imaging software). The % migration was represented 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).

4.3.4: Neurotrophins regulated oligodendrocytes differentiation

Many factors are responsible for the regulation of oligodendrocytes differentiation (Lourenço et al., 2016, Zuchero & Barres, 2013). Growth factors such as neurotrophins are known to regulate many functions like survival, myelination and differentiation in glial cells. They are also involved in the many cascades of glial cells. Of these neurotrophin, NGF enhances the survival of differentiated oligodendrocytes (Cohen et al., 1996) whereas BDNF affects OPC proliferation and development via TrkB (Van't Veer et al., 2009) and promotes CNS myelination via a direct effect on oligodendrocytes (Xiao et al., 2010). However, the neurotrophins’ receptors mediated regulation of OPC differentiation needs to be studied. In the present study, OPC were treated with neurotrophin (NGF) in presence or absence of specific receptor (TrkA) inhibitor for 5 days. Post treatment cell lysate was collected and expression of MBP protein- a marker for mature Oligodendrocyte was checked by western blotting to study the OPC differentiation into mature oligodendrocytes. Results illustrated that NGF treatment did not show any expression change in cells which indicates neutral role of NGF in differentiation of OPC but surprisingly, in TrkA inhibition condition, MBP levels were significantly reduced (fig.4.4) which indicates role of TrkA activation in OPC differentiation.
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Figure 4.4: NGF regulated oligodendrocytes differentiation: OPC were treated with NGF with and without TrkA inhibitor. (A) Typical western blot image of MBP protein in control, only NGF treated and TrkA inhibitor treated group. (B) Graphical representation of MBP protein expression relative to control. MBP Protein expression normalized by respective internal controls protein β-actin. Data were analysed by one-way ANOVA followed by Bonferroni test and presented as mean±SEM of 3 replicate measurements (*p<0.05,) compared with control.

4.4 Discussion:
Central nervous system has oligodendrocytes cells as myelinating cells. An Oligodendrocyte extends many processes, each of which contacts and repeatedly envelopes a stretch of axon and with subsequent condensation of this multi spiral membrane-forms myelin (Bunge et al., 1962, Bunge, 1968). Oligodendrocytes are also called as satellite oligodendrocytes (Penfield, 1932) which may not be directly connected to the myelin sheath. They are perineuronal and may serve to regulate the microenvironment around the neurons (Ludwin, 1997). Before their final maturation involving myelin formation, oligodendrocytes go through many stages of development. Bradl and Lassmann have reported that myelinating oligodendrocytes cells are end product of cell lineage which always undergo in a complex and precisely timed program.
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of proliferation, migration and differentiation into mature oligodendrocytes (Bradl & Lassmann, 2010). Their characterization is often insufficient by morphological criteria alone both in-vivo and in-vitro. A number of distinct phenotypic stages have been identified both in-vivo and in-vitro based on the expression of various specific components (antigenic markers) and the mitotic and migratory status of these cells. Oligodendrocyte precursors originate from neuroepithelial cells in ventricular zone, at very early stages during embryonic life (Curtis et al., 1988, Hardy & Reynolds, 1991, Hardy & Reynolds, 1993, Levine et al., 1993, Pfeiffer et al., 1993). In spinal cord, a ventral region of the neural tube is the initial source of oligodendrocytes. Warf and co-workers (Warf et al., 1991) have shown that Oligodendrocyte precursors arise in the ventral spinal cord and then migrate dorsally during development. Oligodendrocyte progenitors migrate extensively throughout the CNS before their final differentiation into myelin-forming oligodendrocytes (Small et al., 1987). In-vivo and in-vitro data have shown OPCs are actively proliferating and possess migratory properties (Curtis et al., 1988) (Pfeiffer et al., 1993). They proliferate in vitro, in response to growth factors such as fibroblast growth factor (FGF) (Gard & Pfeiffer, 1993, Hardy & Reynolds, 1993, Milner et al., 1997, Raff et al., 1988) and PDGF (Richardson et al., 1988, Tripathi et al., 2017). Many intrinsic and extrinsic molecules such as extracellular matrix (ECM) (Ffrench-Constant et al., 1988) and growth factors (Vora et al., 2011, Decker et al., 2000) regulate oligodendrocytes migration. In the present study, BDNF regulated OPC’s proliferation and migration through TrkB receptor while action of NGF in OPC proliferation and migration was regulated by TrkA receptor. Only BDNF treated OPCs proliferated significantly more compare to control and migrated on average 300% in all time points (24 hrs, 48 hrs and 72 hrs) while TrkB inhibition significantly prevented OPCs migration in in-vitro system. Moreover, Receptor specific inhibitors inhibited the action of neurotrophins and thus neurotrophins regulated (NGF & BDNF) proliferation and migration were inhibited in presence of TrkA and TrkB inhibitors which noticeably suggested that regulation of OPC proliferation and migration, in CNS ,was mediated
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through both the neurotrophins receptors, TrkA and TrkB. Oligodendrocytes are different from other glial cells of the brain such as astrocytes (Peters, 1991), in particular their smaller size, greater density, the absence of intermediate filaments, glycogen in the cytoplasm, and the presence of a large number of microtubules (25 nm) in their processes that is involved in their stability (Lunn et al., 1997). The in-vitro analyses suggested that maturation of oligodendrocytes from the precursor stage to the mature cell is identical in culture, even without neurons, as in intact tissue so, the capacity of Oligodendrocyte progenitors to differentiate into oligodendrocytes is intrinsic to the lineage (Temple & Raff, 1986). In CNS after the migration, oligodendrocytes settle along fiber tracts of the future white matter and then transform into multi-processed pre-oligodendrocytes cells which keep the property of cell division and acquire the marker O4 (Sommer & Schachner, 1981). At this stage, those cells are losing their mitogenic response to PDGF, (Gao et al., 1998, Hart et al., 1989, Pringle & Richardson, 1993) appear sequentially both in-vivo and in-vitro and signify a mature oligodendrocytes and These mature oligodendrocytes express MBP and PLP proteins (Baumann & Pham-Dinh, 2001). Many growth factors have been found to be involved in the proliferation, differentiation, and maturation of the Oligodendrocyte lineage (Cui at al, 2010; Collarini et al, 1991, Dalcq, M et al, 2000). Most of these studies have been performed in-vitro. PDGF is very well known growth factor in this lineage (Butt et al, 1997). Some neurotrophins such as NGF and BDNF are known to involve in the activation of other cells, such as cerebellar granule and schwan cells. In the present study TrkA neurotrophin receptor played an important role in the OPC differentiation lineage. Data revealed that TrkA inhibitor treated OPC suppressed MBP expression compares to control which clearly suggested that regulation of NGF mediated oligodendrocytes differentiation is by TrkA receptor only.

Thus the overall conclusion from this study is that both neurotrophins, NGF and BDNF, regulates oligodendrocytes lineage. BDNF regulates OPC’s migration in CNS through TrkB receptor. Only BDNF treated OPCs proliferated significantly more compare to
control and migrated on average 300% in all time points while TrkB inhibition significantly prevented OPCs proliferation and migration which confirms that BDNF regulates OPC proliferation and migration through TrkB receptor. Similarly, NGF induced OPC proliferation, migration and differentiation through TrkA receptor. Thus, overall results from this objective were that both NGF and BDNF were involved in the regulation of oligodendrocytes lineage from migration to differentiation. BDNF and NGF both regulated proliferation, migration and differentiation of OPCs through TrkB and TrkA receptor, respectively.