Synopsis of the thesis on

Role of BDNF and NGF in Central Nervous System Myelination

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Role of BDNF and NGF in Central Nervous System Myelination

Introduction:

Neurotrophins are class of growth factors which are essential for proper functioning of central nervous system (CNS) such as cell survival, differentiation, cell migration, plasticity and myelination. Involvement of neurotrophins in neural stem cells (NSCs) differentiation has recently started to interest researchers. However, firstly, very little is known in regards to the modulation of cell fate decision of endogenous NSCs and oligodendrocytes precursor cells (OPCs) via appropriate signaling mechanism involving these neurotrophins' action in producing large number of oligodendrocytes and secondly, enhancement of remyelination events following cytokine affected myelin sheath which is a hallmark feature of many demyelinating diseases is still not clear.

Neurotrophins (NTs):

The neurotrophins belong to a class of secreted growth factors that are capable of inducing cell survival, differentiation, or growth. (Allen SJ *et al.,* 2006). They are classified in four types: Nerve growth factor (NGF), Brain derived neurotrophic factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4/5 (NT-4/5). Neurotrophins are initially synthesized as precursor proteins, known as proneurotrophins, and are subsequently cleaved to release the mature neurotrophins proteins. Proneurotrophins are biologically active and signal through a receptor complex consisting of the p75 neurotrophin receptor (p75NTR) and sortilin. Once cleaved, the mature neurotrophins are no longer able to interact with this complex, but rather interact with two distinct classes of transmembrane receptors: the receptor tyrosine kinase tropomyosin-related kinase (Trk), and the structurally unrelated p75NTR, a member of the tumor necrosis factor receptor superfamily. Interactions of neurotrophins with the Trk receptors are selective in nature; NGF binds selectively to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC (Lu *et al.,* 2005). In addition, all the neurotrophins can bind non-selectively to p75NTR (Reichardt 2006, Hempstead 2006, Arevalo *et al.,* 2006). Many pathways are activated through NTs such as ERK, PLC-γ, PI3K which are involved in several important functions of the nervous system (Bucci *et al.* 2014), such as cell migration, proliferation, differentiation as well as in myelination events.

Neural Stem cells (NSCs) and differentiation:

NSCs are dynamic population of cells that contribute new neurons, astrocytes and oligodendrocytes to the brain throughout life and have recently showed potential applications in cell replacement therapy. NSCs are involved in both neurogenesis and gliogenesis. This differentiation of NSCs in astrocytes, oligodendrocytes and neurons is very crucial for neuronal such diseases such as Multiple sclerosis, Alzheimer, Devic's disease and Leukodystrophies to reimburse the normal condition. Proliferation and differentiation of NSCs are not only regulated by the endogenous genes (Artavanis-Tsakonas *et al.,* 1999; Ohsawa *et al.,* 2005), but also closely regulated by various factors including neurotrophic factors (Benoit *et al.,* 2001). Neurotrophic factors existing in the local microenvironment of the brain play important roles in protecting neural functions and repairing brain injuries by supporting the survival of neurons, promoting their growth and differentiation, and 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, 2015). However, very little information is available on the role of neurotrophins in the differentiation of NSCs into oligodendrocytes and the signaling mechanism involved in it.

Oligodendrocyte Progenitor cells: Migration, Proliferation and Differentiation:

Oligodendrocytes are the myelinating cells of the central nervous system (CNS). They are the end product of a cell lineage which has to undergo a complex and precisely timed program of proliferation, migration, differentiation, and myelination to finally produce the insulating sheath of axons (Bradl *et al.,* 2010). Oligodendrocytes migration is very important for myelination as well as remyelination. Role of growth factor, PDGF has been extensively studied in this aspect (Frost *et al.,* 2010). Neurotrophins are also involved in migration of other cells; BDNF stimulates migration of cerebellar granule cells and promote the migration of cortical neurons (Borghesani *et al.,* 2002) and NGF enhances migration of Schwan cells in the brain (Anton *et al.,* 1994). In addition, NGF enhances the survival of differentiated oligodendrocytes (Cohen *et al.,* 1996) whereas BDNF affects OPC proliferation and development via TrkB (Van't Veer A *et al.,* 2009) and promotes CNS myelination via a direct effect on oligodendrocytes (Xiao J *et al.,* 2011).

However, the neurotrophins' receptors mediated signalling mechanisms regulating migration, proliferation and differentiation of OPC population is still unclear.

Central nervous system myelination:

Neuronal signal propagation or action potential in vertebrates is speeded up by the electrical insulation of axons with an ensheathing, specialized glial plasma membrane: myelin sheath. Myelination is done by oligodendrocytes in the CNS and Schwann cells in PNS. In the CNS myelination, early oligodendrocyte progenitors arise from neuroepithelium in the ventral spinal cord and migrate throughout the brain parenchyma (Price *et al.*, 1994) and differentiation into myelinating cells (mature oligodendrocytes). Importance of CNS myelin for normal sensation, cognition, and motor function is obvious considering that myelin-related disorders often affect humans lethally (Sherman DL *et al.*, 2005; Simons *et al.*, 2007). Recent studies identify neurotrophins as important regulators of both peripheral and central myelination; NGF controls axonal receptivity to myelination by Schwann cells in the PNS (Chan *et al.*, 2004) whereas BDNF is a well-studied astrocyte secreted molecule known to promote CNS myelination (Xiao J *et al.*, 2011). During inflammation and in chronic injury, myelination is affected and myelin proteins' level goes down (Thomson *et al.*, 2008). At this particular time exogenous molecules like growth factors try to recover the normal condition for survival of CNS myelinating cells, oligodendrocytes. However, knowledge on role of neurotrophins and their receptors in myelination recovery in cytokine affected (inflammation condition) myelination is still vague.

The study aims to provide valuable information on the mechanisms governing myelination involving neurotrophins. Some relevant potential therapeutic strategies can be developed based on understanding of neurotrophin mediated NSCs differentiation into oligodendrocytes, migration of OPCs and remyelination in cytokine affected myelination in developing brain. Identification of the downstream signaling pathways of neurotrophins involved in above mentioned tasks will further enhance our understanding of molecular and cellular mechanisms of neurotrophins involved in myelination.

Hence the objectives of the present study were defined as follows-

1. To determine the role of BDNF, NGF and their receptors in the regulation of Neural Stem Cell (NSC) differentiation into oligodendrocytes.

2. To identify the involvement and signalling mechanism of BDNF and NGF in Oligodendrocyte Progenitor Cells (OPCs) proliferation, migration and differentiation.

3. To identify the involvement of BDNF and NGF in remyelination in cytokine affected myelination**.**

Results and Discussion:

Objective 1.: To determine the role of BDNF, NGF and their receptors in the regulation of Neural Stem Cell (NSC) differentiation into oligodendrocytes.

Primary culture of Neural stem cells (Neurosphere culture) was optimized from newly born rat pups (P0) as described by Pacey et al, 2006. Further, the Neural stem cells (NSCs) culture was characterized by immunocytochemistry using antibodies against specific markers Nestin, PDGFR-α, GFAP and Tuj1. To study the role of Neurotrophins (NGF and BDNF) in oligodendrogenesis, NSCs were treated with different doses of NGF and BDNF and cell survival, proliferation and differentiation assays were carried out. Results of MTT assay (cell survival assay) revealed that exogenous NGF and BDNF were not affecting cell viability of NSCs while the results of BrdU cell proliferation assay showed that NGF (10ng/ml) up regulated NSCs' proliferation but not BDNF. For differentiation study, NSCs were treated with NGF and BDNF (10 and 50ng/ml, respectively) for 10 days, whole cell lysate and RNA was isolated and Western blotting and real time PCR for Myelin basic protein (MBP), mature oligodendrocytes marker, was carried out. Data of western blot and real time suggested that NGF and BDNF both triggered oligodenrogenesis from NSCs. On the other hand GFAP (Astrocytes marker) and Tuj1 (Neuron marker) expression was not altered as compared to control. Surprisingly, higher dose (50ng/ml) of neurotrophins (NGF and BDNF both) upregulated GFAP expression which indicated optimum dose (10ng/ml) of neurotrophins allowed NSCs to differentiate into

oligodendrocytes only while higher dose triggered glial cells (Astrocytes and Oligodendrocytes lineage both) lineage. Neurotrophins activate three different signaling pathways viz. PLC-γ, AKT and ERK pathways, amongst which only ERK plays a role in the cell differentiation cascade. Now, to confirm neurotrophins mediated regulation of differentiation, phosphorylation of ERK was monitored and results showed that activation of ERK was higher in NGF and BDNF treated cells compared to control, indicating that ERK pathway was activated by neurotrophins during NSC differentiation. For further confirmation, specific inhibitors for neurotrophin receptors (TrkA receptor for NGF and TrkB receptor for BDNF) were used. Cells were pre-treated with inhibitors (1μM, GW 441756, TrkA inhibitor and 10μM ANA12-CAS, TrkB inhibitor) followed by neurotrophins treatment for next 10 days for differentiation. Inhibitor studies confirmed that action of NGF and BDNF for differentiation mediated by TrkA and TrkB only. Protein expression of MBP was significantly downregulated in Trk inhibitors' group compared to only Neurotrophins group which indicated specific receptor mediated role of NGF and BDNF in oligodendrocytes differentiation. ERK activation was downregulated in inhibitor treated group compared to only neurotrophins suggesting TrkA and TrkB mediated ERK activation during NSC differentiation. Over all conclusion from first objective was that both NGF and BDNF neurotrophins promoted oligodendrocytes differentiation from NSC and action was mediated by Trk receptors (TrkA and TrkB) in which ERK pathway played a important role for oligodendrocytes differentiation.

Objective 2.: To identify the involvement and signalling mechanism of BDNF and NGF in Oligodendrocyte Progenitor Cells (OPCs) proliferation, migration and differentiation.

Oligodendrocytes precursor cells (OPCs) were isolated from rat pups (0-2 day old) (Chen et al., 2007). Migration study was carried out with neurotrophins (NGF and BDNF) specific groups. Groups were as; (1) Control, NGF (10ng/ml), TrkA inhibitor + NGF; (2) Control, BDNF (10ng/ml), TrkB inhibitor + BDNF. Time period for migration study was 24 hrs, 48 hrs and 72 hrs. OPCs were seeded on PLL coated cell culture well and wound scratch assay was performed at above mentioned time period for migration study. Distance migrated by cells was calculated using *NIS software*. Results suggested that BDNF regulated OPC's migration in CNS through TrkB receptor. BDNF treated OPCs migrated on average 250% in all time points while TrkB

inhibition significantly prevented OPCs migration which confirms that BDNF regulates OPC migration through TrkB receptor. Similarly, NGF induced OPC migration through TrkA receptor. Thus, over all conclusions from this objective was that both NGF and BDNF were involved in migration of OPCs through TrkA and TrkB receptor, respectively.

Objective 3.: To identify the involvement of BDNF and NGF in remyelination in cytokine affected myelination.

Myelinating spinal culture was optimized from E13.5 day pregnant mice followed by Thomson's protocol (Thomson et al, 2008). This *in vitro* system provides a platform to underline the CNS myelination mechanism. Using this system, cultures were initially treated with TNF-α (DIV 15-23) with different doses (1, 10 and 20ng/ml) to induce inflammation which further lead to demyelination. To confirm the demyelination, TNF-α treated myelinating culture was fixed for immunocytochemistry of MBP protein (Myelin protein), wherein results showed significantly decreased level of MBP in 20ng/ml TNF-α dose. Same result was observed in western blot also which indicated 20ng/ml TNF-α affects myelination in myelinating spinal cord culture. To underline the in depth role of TNF-α in demyelination, apoptotic protein (Caspase-3) and TNF-α receptors (TNFR1 and TNFR2) expression were studied and data revealed that TNF-α affected myelination rather than cell death and action of TNF-α was mediated by TNFR1 receptor. To confirm the remyelination potential of neurotrophins, cells were treated with TNF-α along with BDNF and results revealed presence of BDNF helped to sustain the expression of myelin protein (MBP) which indicated BDNF plays a vital role in CNS myelination regulation in inflammatory condition.

Conclusion:

In the current study, we observed that both NGF and BDNF neurotrophins regulate CNS myelination through oligodendrocytes differentiation from NSCs, OPC migration and remyelination in cytokine affected myelination and action of NGF and BDNF was mediated by TrkA and TrkB receptors respectively which suggested that NGF and BDNF can be considered as therapeutic agents for demyelination diseases such as Multiple sclerosis, Alzheimer, Devic's disease.

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Animal ethical statement:

All the mentioned studies were approved by institutional animal ethics committee (IAEC). All protocols (ZD/02/2015, 2016, 2017) were approved from Animal House Facility, Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Vadodara.

Publications:

1. J Langhnoja, L Buch, P Pillai. 2015/8/1. **"Modulatory effects of tumor necrosis factor α on neurotrophins in myelinating spinal cord and dorsal root ganglion cultures".** JOURNAL OF NEUROCHEMISTRY. Volume 134. Pages 201201. Publisher WILEYBLACKWELL.

2. L Buch, J Langhnoja, P Pillai. 2015/8/1. **"Role of astroglial methyl-CpG-binding protein 2 in central nervous system myelination.** JOURNAL OF NEUROCHEMISTRY". Volume 134. Pages 283283. Publisher WILEYBLACKWELL.

3. 17β-estradiol modulates NGF and BDNF expression through ERβ mediated ERK signaling in cortical astrocytes. (Under review)

4. Neurotrophomodulatory effect of TNF-α in rat cortical astrocytes through NF-κB pathway. (Under review)

5. Role of Brain derived neurotrophins factor, Nerve growth factor and their receptors in the regulation of Neural Stem Cell differentiation into Oligodendrocytes. (Under preparation)

6. Insulin Receptor regulates Neurotrophins and their receptors levels for neural stem cells differentiation: In-Vitro study. (Under preparation)

Workshop/Conference/Seminar

- Poster presentation entitled "Cellular mechanism underlying modulatory effects of 17β- estradiol in neurotrophins expression in rat cortical astrocytes" presented at the 33rd Annual Meeting of the Society for Reproductive Biology and Comparative Endocrinology (SRBCE): International Conference on Bioactive Chemicals for Reproduction and Human Health, Davangere University, Karnataka, February 26th-28th, 2015. **(First author, Presenter)**

- Poster presentation entitled "Modulatory Effects of Tumor Necrosis Factor α on neurotrophins in Myelinating Spinal Cord and Dorsal Root Ganglion cultures" presented at the 25th Biennial meeting of the International Society of Neurochemistry (ISN) and Asian Pacific Society of Neurochemistry (APSN), Cairns, Australia, August 23rd-27th, 2015 **(First author, Presenter)**

- Poster presentation entitled "Role of Astroglial Methyl-CpG-Binding Protein 2 in Central Nervous System Myelination" presented at the 25th Biennial meeting of the International Society of Neurochemistry (ISN) and Asian Pacific Society of Neurochemistry (APSN), Cairns, Australia, August 23rd-27th 2015 **(Co-Author)**

- Poster presentation entitled "Cross-talk between Insulin Receptor and Neurotrophins in neural stem cells differentiation" presented at the 34th Annual Meeting of Indian Academy of Neurosciences (IAN), National Brain Research Centre, Manesar, INDIA, October 19th–21st 2016 **(First Author, Presenter)**

- Hands on Training One Day Workshop on **Neurostereology and Assessment of Pain in Animals and Humans,** Department of Anatomy and Physiology, All India Institute of Medical Sciences (AIIMS), New Delhi, INDIA, 18TH October 2016.

- Poster presentation entitled "Role of nerve growth factor and brain derived neurotrophic factor in regulation of central nervous system myelination" presented at the Annual meeting (47th) of Society of Neuroscience (SFN), Washington (DC), USA, November 11th – 15th , 2017 **(First author, Presenter)**

Awards:

- Bagged **Prof. P. Chinoy award (first prize)** for best poster presentation in the 33rd Annual Meeting of the Society for Reproductive Biology and Comparative Endocrinology (SRBCE), February 2015.

- **DBT-CTEP Travel Grant award** to attend the 25th Biennial meeting of the International Society of Neurochemistry (ISN) and Asian Pacific Society of Neurochemistry (APSN), Cairns, Australia, August 2015.

- **Best Poster award** in 34th Annual Meeting of Indian Academy of Neurosciences (IAN), National Brain Research Centre, Manesar, INDIA, October 2016.

- **DST-SERB Travel Grant award** to attend the 47th meeting of Society for Neuroscience (Sfn), Washington DC, USA, November 2017

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