Chapter 5
To identify the involvement of BDNF and NGF in remyelination in cytokine affected myelination

5.1. Introduction:

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 (Webb *et al.*, 1995) and differentiate into myelinating cells (mature oligodendrocytes). Importance of CNS myelination for normal sensation, cognition, and motor function is obvious, considering that myelin-related disorders often affect humans lethally (Sherman & Brophy, 2005; Simons & Trotter, 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 *et al.*, 2010, Fulmer *et al.*, 2014). 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 remyelination involving neurotrophins. Some relevant potential therapeutic strategies can be developed based on understanding of neurotrophin mediated remyelination in cytokine affected myelination in developing brain.

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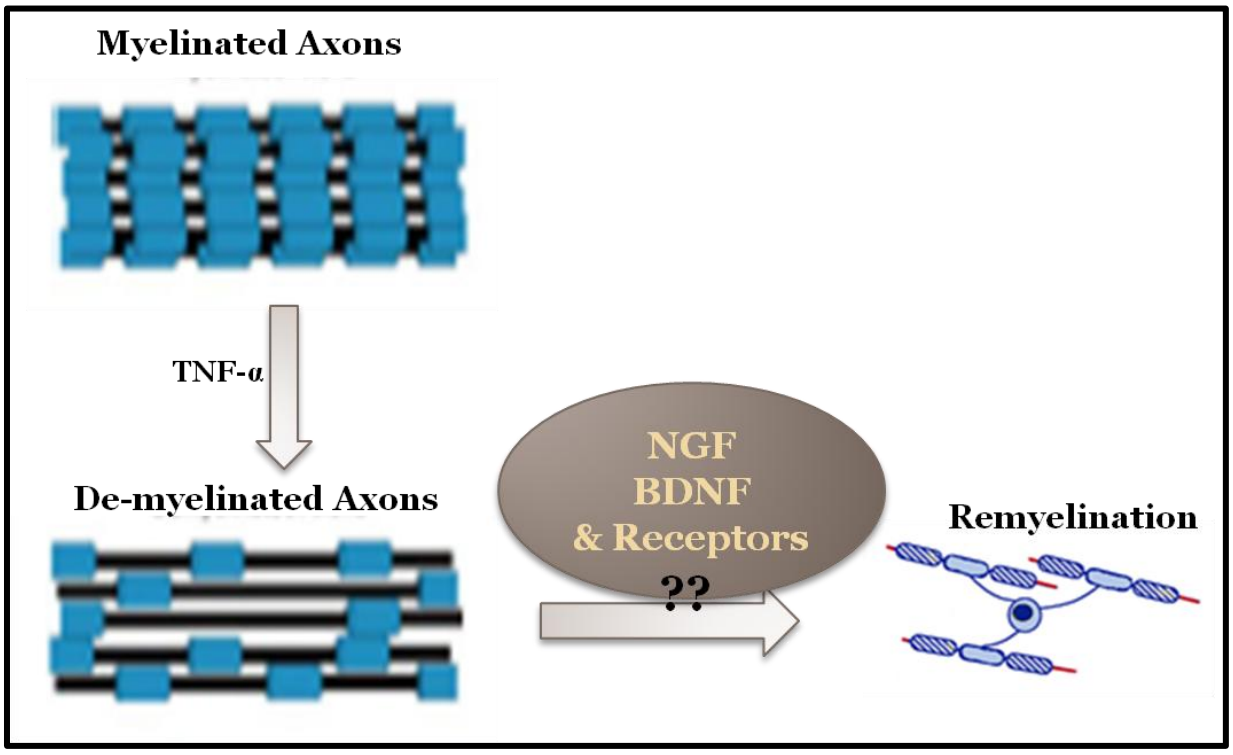

5.2. Strategy of work:

The whole work was divided in three strategies

A. Establishment of Myelinating spinal cord culture and Characterization

B. TNF- α induced demyelination in Myelinating culture

C. Study the remyelination potential of BFNF and NGF for TNF- α induced demyelination

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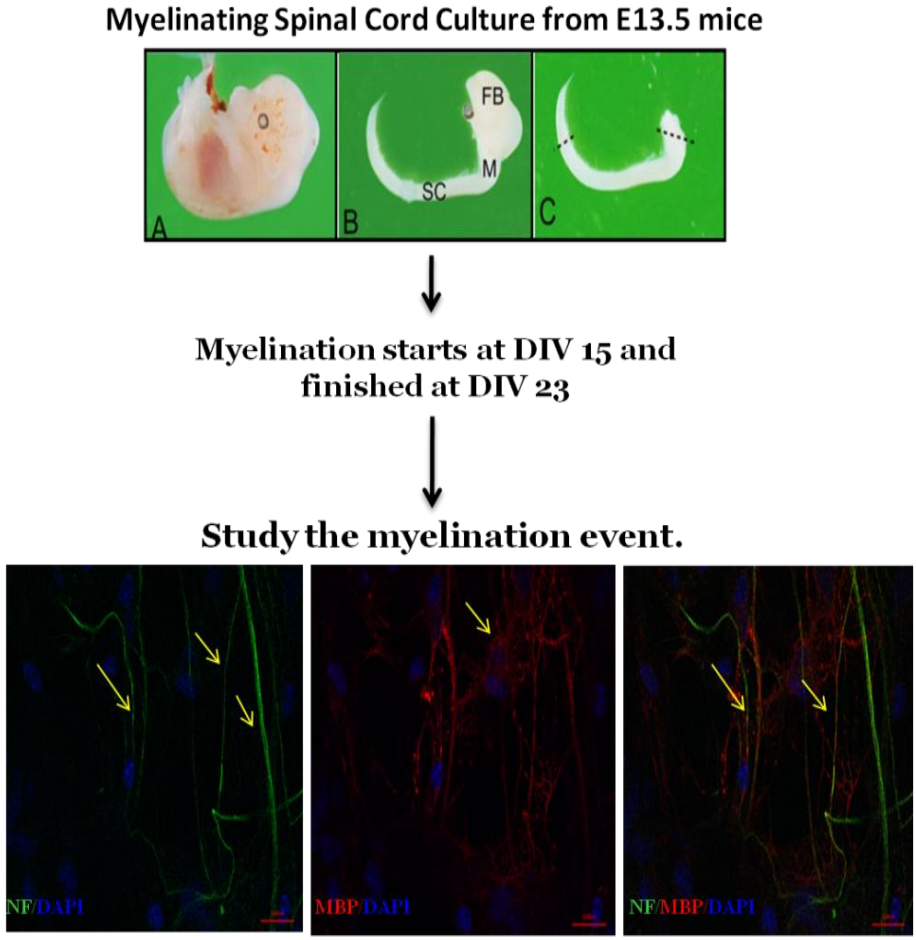
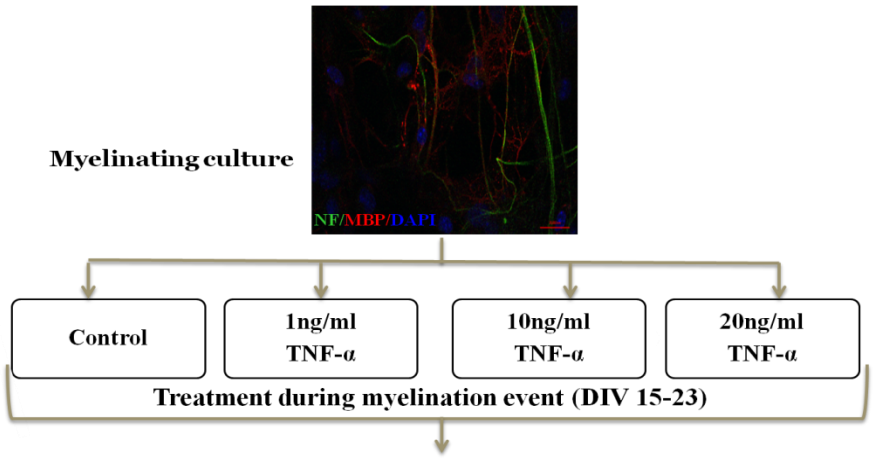
A. Establishment of Myelinating spinal cord culture and Characterization

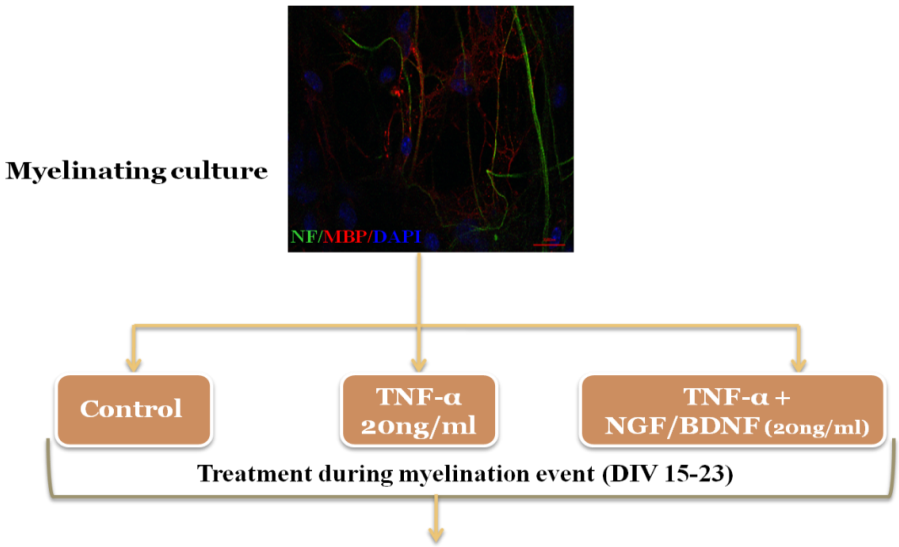


B. TNF- α induced demyelination in Myelinating culture



- Apoptotic marker activation and TNF-α signaling for demyelination.
- Expression of Myelin Basic Proteins (MBP) by Real time PCR , western blot and Immunocytochemistry (ICC).
- Myelin protein concentration in myelin sheath.

C. Study the remyelination potential of BFNF and NGF for TNF- α induced demyelination



- Studied the expression of Myelin Basic Proteins (MBP) by western blot and Immunocytochemistry.
- Studied myelin protein concentration in myelin sheath.

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5.3 Results:

5.3.1: Isolation and characterization of Myelinating spinal cord culture

Myelinating Spinal Cord culture was established from E13.5 time pregnant mice following Thomson *et al.*, 2008 protocol (Thomson *et al.*, 2008). Wild-type balb/c female mice were time-mated and embryos were collected on embryonic day E13.5. 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 and the embryos were extracted. Foetuses were decapitated at about 3mm rostral to the cervical flexure along with 8-10mm of spinal cord which was transferred to sterile DMEM without serum. Remaining meninges were removed and tissue was digested using 1X trypsin and 1% collagenase and incubated at 37^0C for 20 minutes. Post incubation, SD solution was added and tissue fragments were dissociated by gentle trituration. Plating medium (PM) 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 autonomously, myelination event starts at Day *in-vitro* 15 (DIV 15) and by DIV 23 a vast majority of all neurons gets myelinated. This time window of DIV 15 to DIV 23, therefore, was selected for remyelination study in the present work. Characterization of culture was done using cell-specific markers in DIV 20 culture; neurons stained with NF and oligodendrocytes with MBP. Myelinated axons were MBP positive and indicted by yellow colored arrows in Figure 5.1.

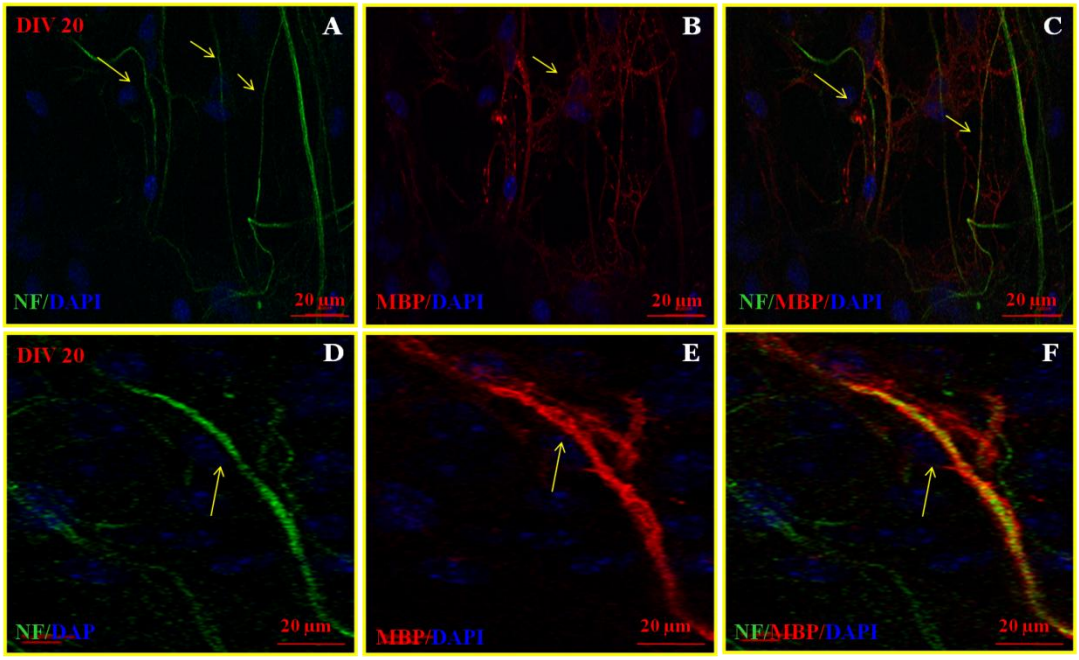

Figure 5.1: Characterization of spinal cord culture of time pregnant 13.5 day mice embryo yields predominantly Neurofilament [NF] and Myelin Basic Protein [MBP] positive cells at DIV 20. *63X Immunofluorescent microscopy using Mouse Anti-NF (Green) and Goat Anti-MBP (Red) monoclonal antibody visualized with Anti-Mouse FITC and Anti-Goat TRITC secondary antibody respectively. (A) NF, green, positive neurons, (B) MBP, red, positive oligodendrocytes, (C) Merge image of myelin wrapped axon. (D-F) A portion of myelinated axon. Yellow colored arrows directing the NF positive neurons and MBP positive myelinating oligodendrocytes as well as myelinated axon of neurons. DAPI stain for nucleus identification. Scale Bar= 20µm.*

5.3.2: TNF-α induced demyelination in myelinating culture: Dose dependent study of TNF-α on myelin protein levels, apoptotic markers and TNF-α receptors in the event of demyelination

TNF-α, an inflammatory cytokine is know molecule involved in inflammation diseases (Probert *et al.*, 1997, Olmos & Lladó, 2014). TNF-α cascade activate through two distinctive receptors; TNFR1 and TNFR2. TNFR1 receptor is involved in apoptosis and

necrosis while TNFR2 participates in the cell survival function of TNF-α (Sedger & McDermott, 2014; Probert, 2015). TNF-α is a well known trigger of apoptosis and necrosis of oligodendrocytes in both *in-vivo* and *in-vitro* conditions (Selmaj & Raine, 1988, Robbins *et al.*, 1987, Jurewicz *et al.*, 2005). In the present study, the remyelination potential of neurotrophins was studied in myelinating culture, where cells were initially treated with TNF-α in dose dependent manner (1ng/mL, 10ng/mL and 20ng/mL) for demyelination. As discussed above, in myelinating spinal cord culture, myelination event starts at DIV 15and continues up to DIV 23. Thus, the time period for TNF-α treatment selectedwas DIV15 to DIV 23. Culture was grown in DfM medium till DIV 14; followed by TNF-α treatment in DMEM medium from DIV 15 to DIV 23. Post treatment, cell lysate was collected for the assessment of TNF-α activated signaling and myelin protein concentration. Transcript (Fig. 5.2 A) and protein (Fig. 5.2 B) levels of MBP clearly suggest that 20ng/mL concentration of TNF-α significantly down regulated MBP expression in both mRNA as well as protein levels. MBP is one of the major myelin protein present in CNS myelin sheath. In addition, myelin concentration was also analyzed by myelin extraction procedure (Described in Chapter 2, materials and methods) and found TNF-α (20ng/mL) down-regulated myelin protein concentration in myelin sheath (Fig.5.2.C). However, TNF-α did not affect pro-casp 3 protein levels (Fig.5.2.D) which suggests that the effect of TNF-α does not affect the cell viability but only the myelin protein concentration. These observations were in sync with the expression of TNFR1 and not TNFR2 thus indicating the demyelination occurred by TNFR1 receptor signaling and not by involvement of TNFR2.Immunocytochemical analysis confirmed the event of demyelination with 20ng/mL TNF-α induction, wherein it is clearly seen that neuron (NF-green) is wrapped with oligodendrocytes (MBP-red) in control panel cells (Fig 5.2.E) while in 20ng/mL TNF-α induction, there is complete disruption of the myelination (co-localization of NF and MBP) indicating demyelination in culture.

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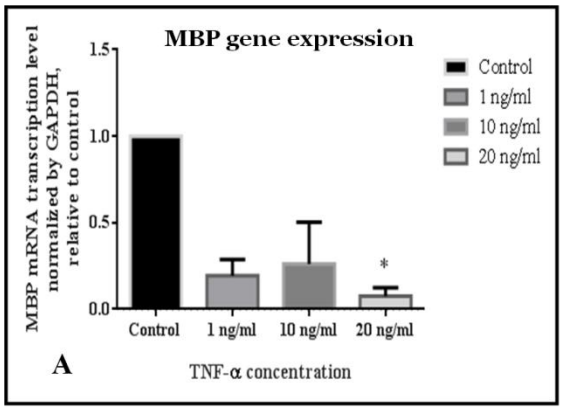
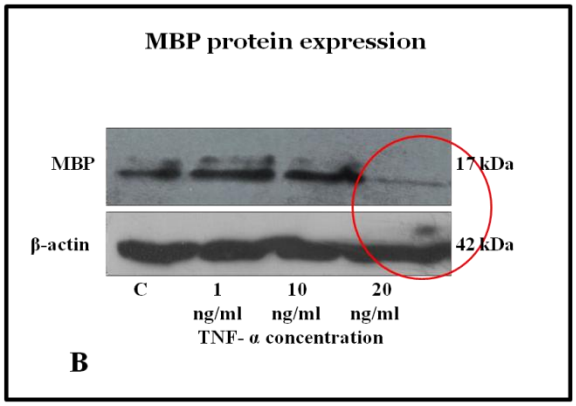
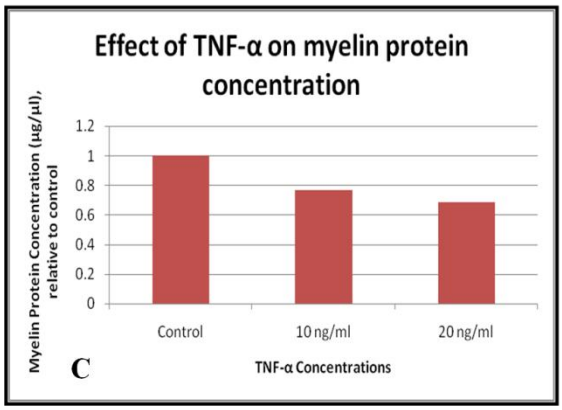
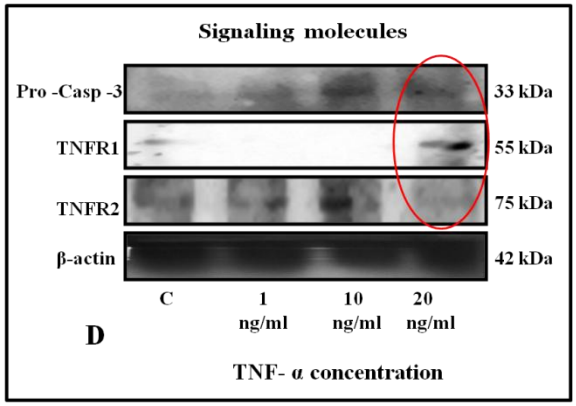








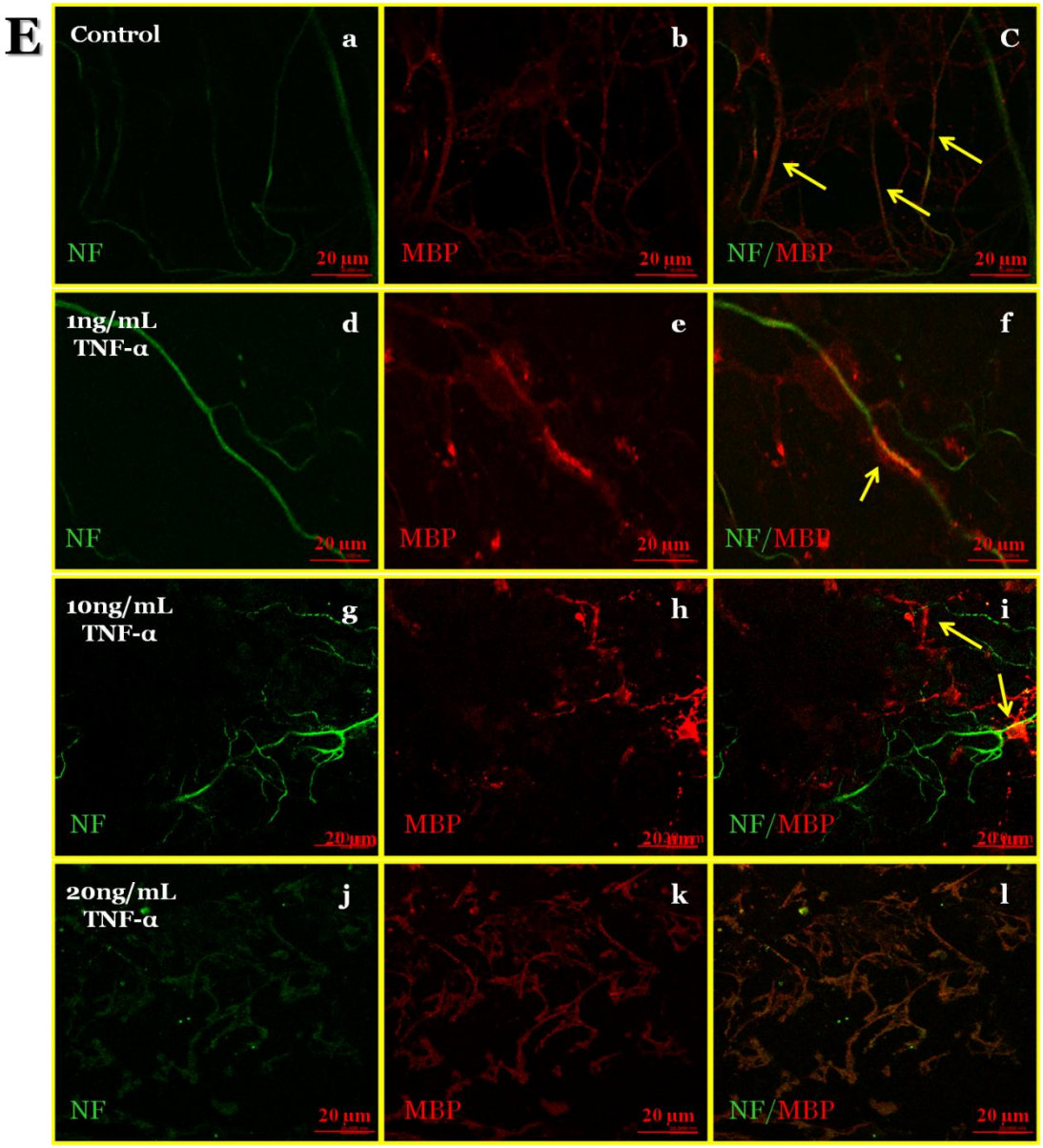


Figure 5.2: TNF-α induced demyelination in myelinating culture: *culture were treated with 1, 10 and 20ng/mL of TNF-α from DIV15 to DIV23 for demyelination induction. Yellow colored arrows indicating myelinating portion where oligodendrocytes formed proper myelin sheath around the axon of neuron. Myelin protein were studied after post treatment (A) Real time PCR was performed to study the validate change in transcripts level of MBP. (B) Western blot of MBP protein in dose depended TNF-α treatment. (C) Myelin protein concentration from extracted myelin by Qubit instrument. (D) Western*

*blot of pro-casp3 along with TNFR1 and TNFR2 for TNF-α activated signaling. (E) Immunocytochemistry of NF and MBP co-localization for myelination study, NF- green, MBP- red, [a-c]: control group of TNF-α treatment, [d-f]: 1ng/mL TNF-α, [g-i]: 10ng/mL TNF-α, [j-l]: 20ng/mL TNF-α. Results were evaluated by one-way ANOVA followed by Bonferroni test and expressed as Mean ± SEM of three independent experiments (***p<0.001, **p < 0.01, *p<0.05).*

5.3.3: Dose dependent effect of TNF-α on Neurotrophins

Neurotrophins are essential growth factor for CNS function. During inflammation, levels of these neurotrophins get modulated. In the present study,myelinating culture was treated with TNF-α (1, 10, & 20ng/mL) from DIV15 to DIV23 followed by assessing the transcript and protein expression of BDNF and NGF. BDNF mRNA expression was significantly decreased post treatment with all concentrations of TNF-α, with 20ng/mL showing the maximum decrease (Fig.5.3A). BDNF protein levels also had similar observations with transcript levels and maximum reduction was found at 20ng/mL TNF-α concentration(Fig.5.3C). There was also decline in BDNF protein levels in 10ng/ml concentration; however the results were not significant. NGF mRNA expression was also significantly reduced with treatment of 20ng/mL concentration of TNF-α (Fig.5.3A). However there was no significant change at 1ng/mL and 10ng/mL concentrationof TNF-α. Similarly the protein expression of NGF was also affected by TNF-α wherein 20ng/mL showed maximum decline in the protein expression but there was no significant change at lower concentrations (1ng/mL and 10ng/mL) (Fig.5.3C)

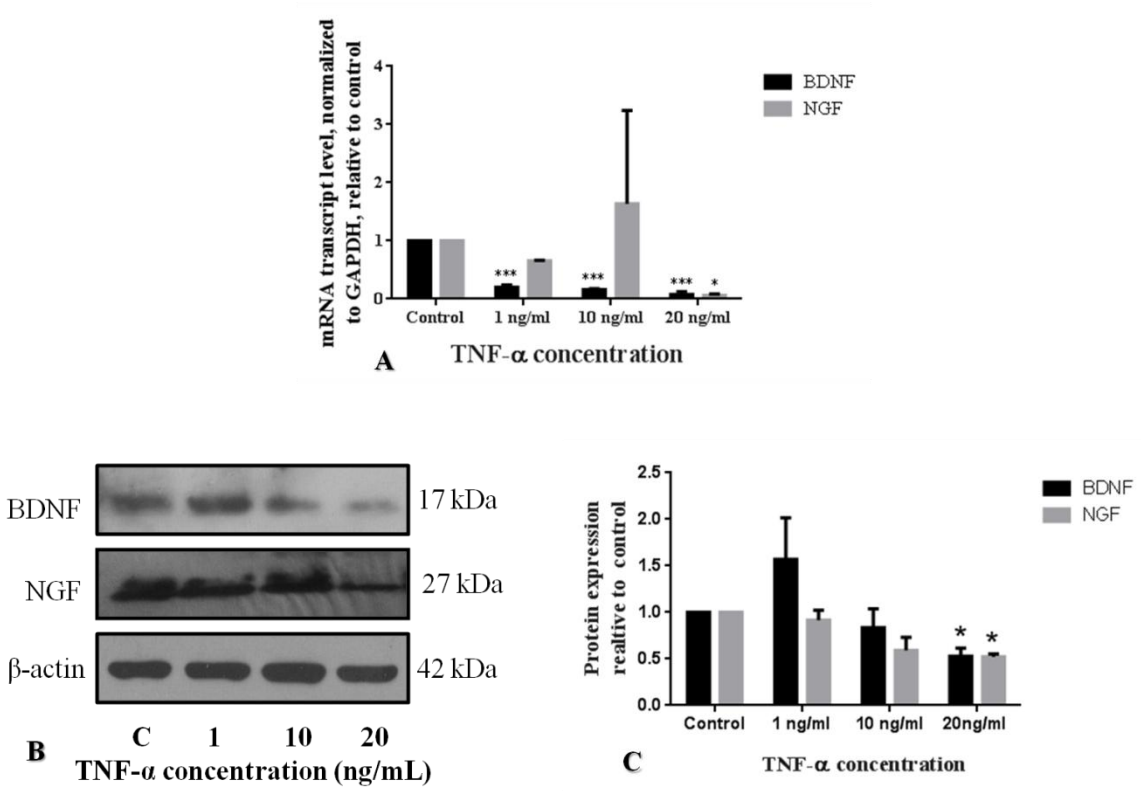
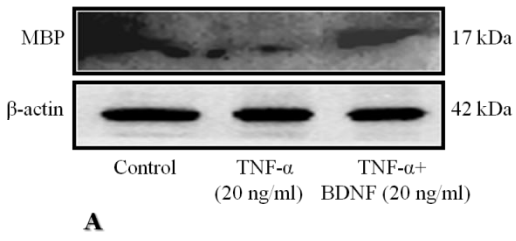


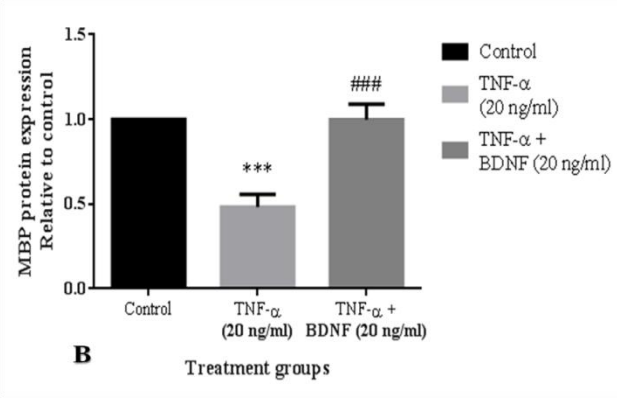
Figure 5.3: **Dose dependent effect of TNF-α on Neurotrophins*:*** *Cells were treated with 1ng/mL, 10ng/mL and 20ng/mL of TNF-α during DIV15 to DIV23. A) Graphical representation of mRNA expression of bdnf and ngf gene, normalized to gapdh and relative to control. B) Representative western blot image of BDNF and NGF proteins level. C) Graphical representation of BDNF and NGF protein levels, normalized to β-actin and relative to control. Results were evaluated by one-way ANOVA followed by Bonferroni test and expressed as Mean ± SEM of three independent experiments. (***p<0.001, **p < 0.01, *p<0.05).*

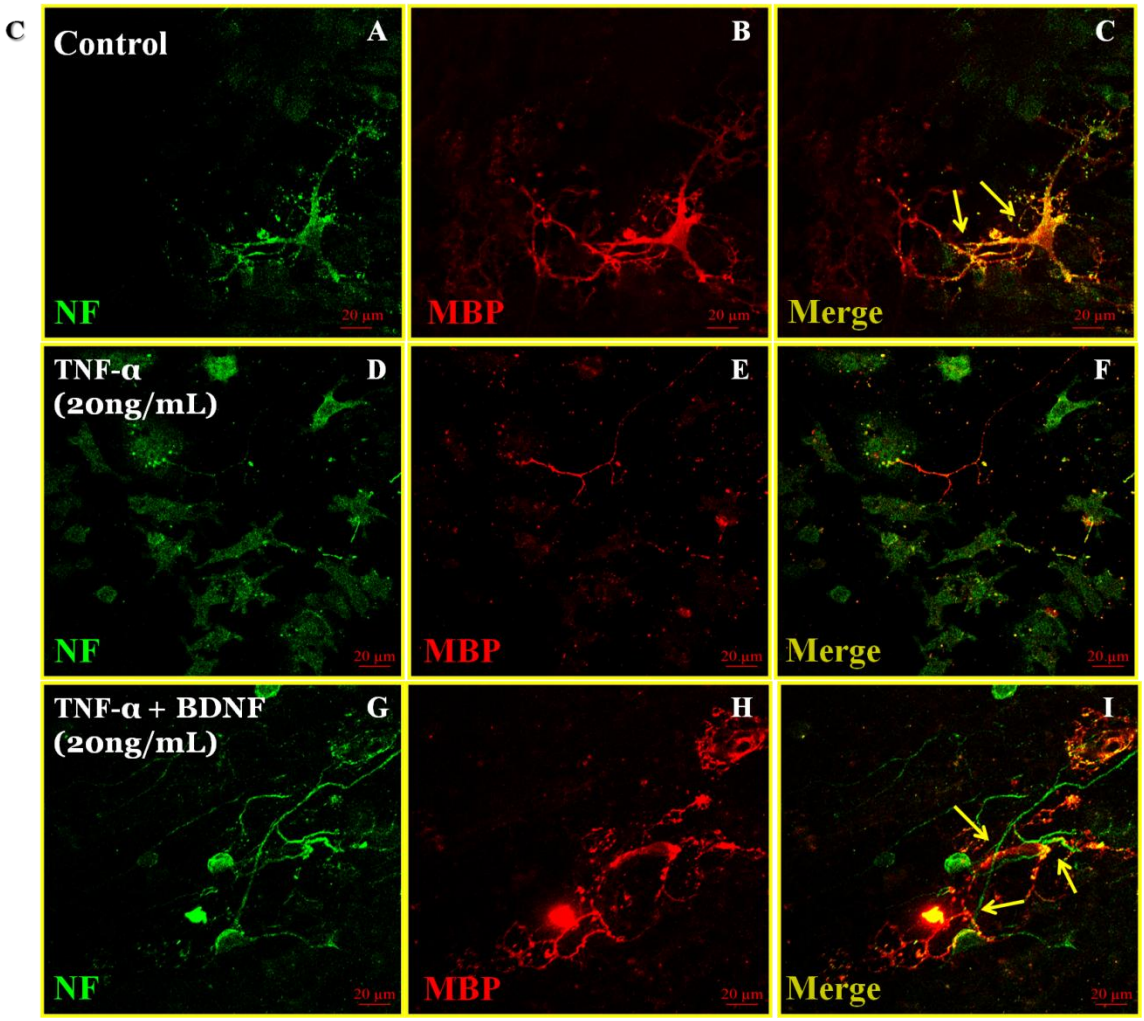
5.3.4: Study of remyelination potential of Neurotrophins:

5.3.4.1: Role of BDNF in regulation of myelination in TNF-α induced demyelination

Myelinating culture cells were treated in following way: a) control, b) TNF-α (20ng/mL), c) TNF-α + BDNF (20ng/mL). Treatment was given from DIV15 to DIV 23. Post treatment cell lysate was collected for western blot study of MBP protein as well as cells were also immunostained for myelination study. Myelin protein concentration was also analyzed in myelin extract of above treated groups. Results revealed that MBP protein levels showed marked decline when cells were treated with TNF-α alone however when treated with BDNF along with TNF-α there was significant up-regulation of MBP levels compare to only TNF-α treatment group (Fig.5.4.1B). These observations were confirmed by ICC wherein myelination (NF & MBP co-localization) was clearly visible in control cells, however the group treated with TNF-α alone showed disrupted myelination while BDNF treated group showed near to normal myelinated neurofilament (merge image fig. 5.4.1C) which indicated preventive role of BDNF in TNF-α induced demyelination. Myelin protein concentration isolated from the myelin sheath of cultured cells demonstrated that BDNF treatment along with TNF-α elevated myelin protein concentration compared to only TNF-α treated group which suggests BDNF can prevent damage caused by TNF-α (Fig. 5.4.1D). Thus current study suggested protective role of BDNF in TNF-α induced demyelination in CNS myelinating (Fulmer et al, 2014).







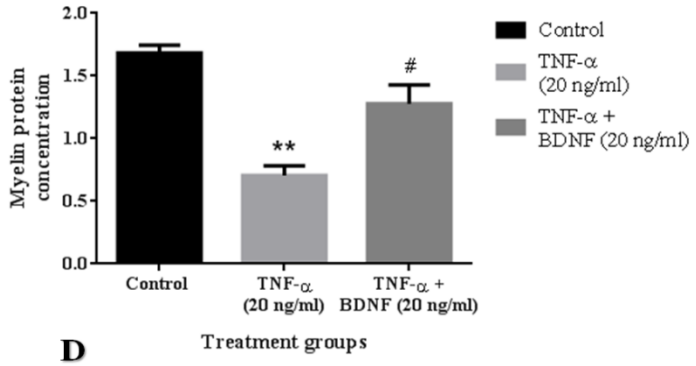
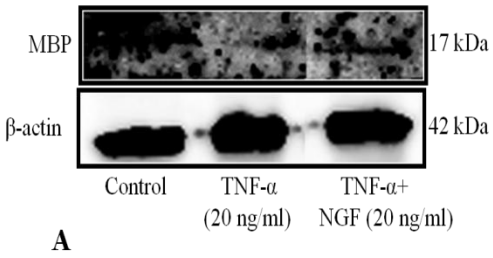


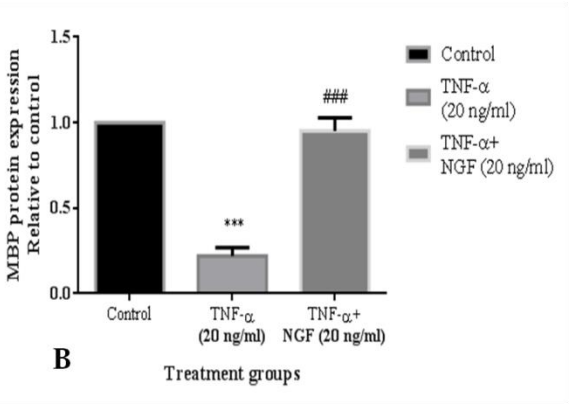
Figure 5.4.1: BDNF potential for remyelination in TNF-α induced demyelination in myelinating culture: *culture was treated with Control, TNF-α (20ng/mL) and BDNF*

*(20ng/mL) + TNF-α from DIV15 to DIV23. Yellow colored arrows indicating myelinating portion where oligodendrocytes formed proper myelin sheath around the axon of neuron. Myelin protein (MBP) were studied after post treatment (A) Representative western blot image of MBP protein level in treatment group. (B) Graphical representation of MBP protein levels, normalized to β-actin and relative to control. (C) Immunocytochemistry of NF and MBP co-localization for remyelination study, NF- green, MBP- red, [A-C]: control group of treatment, [D-F]: 20ng/mL TNF-α, [G-I]: 20ng/mL TNF-α + 20ng/mL BDNF. (D) Myelin protein concentration from extracted myelin by Qubit instrument. Results were evaluated by one-way ANOVA followed by Bonferroni test and expressed as Mean ± SEM of three independent experiments. P values compare to control (***p<0.001, **p < 0.01, *p<0.05). P values compare to TNF-α (20ng/mL) (###p<0.001, ##p < 0.01, #p<0.05).*

5.3.4.2: Role of NGF in regulation of myelination in TNF-α induced demyelination

To study the role of NGF in regulation of CNS myelination, myelinating culture cells were treated with following groups: a) control, b) TNF-α (20ng/mL), c) TNF-α + NGF (20ng/mL). Treatment was given to DIV15-23. Post treatment MBP expression was determinedby western blot and immunostaining for myelination analysis. The results of NGF treatment along with TNF-α are in accordance with BDNF treated cells. There was significant decrease ($p< 0.001$) in MBP levels when the cells were treated with TNF-α alone and the addition of NGF along with TNF-α significantly ($p<0.001$) prevented the disruption of MBP as analyzed with immunoblotting (Fig.5.4.2B). The results of ICC further confirmed the preventive role of NGF in the culture wherein control group cells showed ideal myelination (Fig.5.4.2C) and TNF-α treated cell showed reduced or disruptive myelination, however the cells treated with NGF and TNF-α combine showed improved myelination on neurofilament compared to TNF-α treatment alone and near normal to control cells. Thus it is confirmed that NGF in the culture prevents the TNF-α induced damage of the myelinating cells.


MBP
17 kDa
β-actin
42 kDa
Control
TNF-α (20 ng/ml)
TNF-α+ NGF (20 ng/ml)
A


MBP protein expression Relative to control
1.5
1.0
0.5
0.0
***
###
Control
TNF-α (20 ng/ml)
TNF-α+ NGF (20 ng/ml)
Treatment groups
B

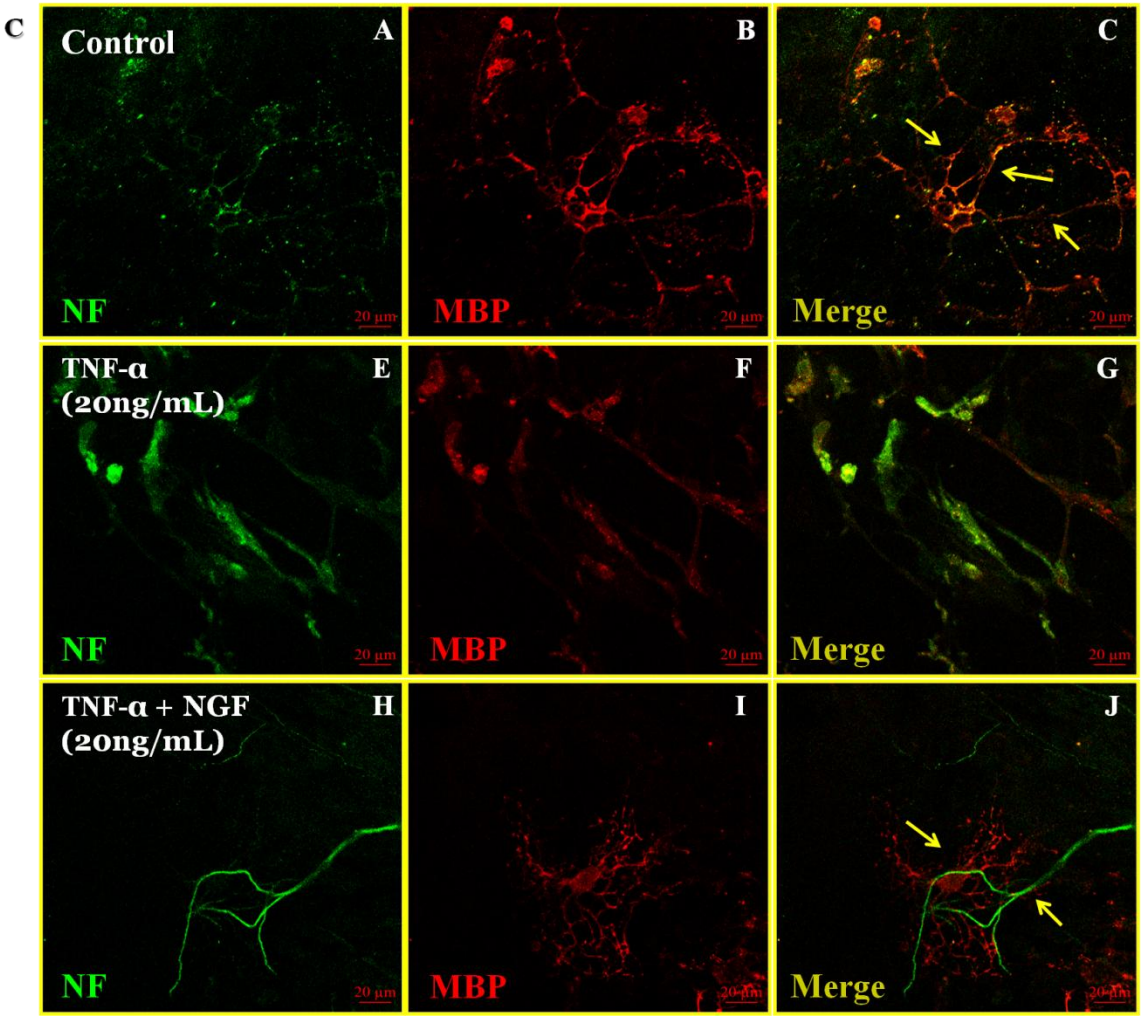

C
Control
A
NF
20 µm
B
MBP
20 µm
C
Merge
20 µm
TNF-α (20ng/mL)
E
NF
20 µm
F
MBP
20 µm
G
Merge
20 µm
TNF-α + NGF (20ng/mL)
H
NF
20 µm
I
MBP
20 µm
J
Merge
20 µm

Figure 5.4.2: NGF potential for remyelination in TNF-α induced demyelination in myelinating culture: *culture was treated with Control, TNF-α (20ng/mL) and NGF (20ng/mL) + TNF-α from DIV15 to DIV23. Yellow colored arrows indicating myelinating portion where oligodendrocytes formed proper myelin sheath around the axon of neuron. Myelin protein (MBP) were studied after post treatment (A) Representative western blot image of MBP protein level in treatment group. (B) Graphical representation of MBP protein levels, normalized to β-actin and relative to control. (C) Immunocytochemistry of NF and MBP co-localization for remyelination study, NF- green, MBP- red, [A-C]: control group of treatment, [D-F]: 20ng/mL TNF-α, [G-I]: 20ng/mL TNF-α + 20ng/mL BDNF. Results were evaluated by one-way ANOVA followed by Bonferroni test and expressed as Mean ± SEM of three independent experiments. P values compare to control (***p<0.001, **p < 0.01, *p<0.05). P values compare to TNF-α (20ng/mL) (###p<0.001, ##p < 0.01, #p<0.05).*

5.4: Discussion:

CNS Myelin is the multi-layered membrane that surrounds most axons and is produced by oligodendrocytes. Single oligodendrocytes can wrap multiple axons, and the thickness and lengths of individual myelin sheaths can vary considerably (Michalski & Kothary, 2015, Baumann & Pham-Dinh, 2001). Myelination consists of the formation of a membrane with a fixed composition and specific lipid-protein interactions. Transcripts encoding some myelin proteins, notably MBP, are transported through oligodendrocyte processes and translated near the point of axon contact (Ainger *et al.*, 1993). Myelin production functions in close synchronization with the cytoskeletal rearrangements in the myelinating oligodendrocyte which produces the myelin membrane and ensheaths axons. Here, in current study, for understanding myelination mechanism and myelin sheath biochemistry we established the myelinating culture (Thomson *et al.*, 2008) in which myelinating event start at day *in-vitro* 15 and all the axons were getting myelinated around at day *in-vitro* 23. Culture was positive for Neurofilament (NF, neuron marker) and Myelin basic protein (MBP, oligodendrocytes cells and myelination marker)

expression. CNS myelination is affected by many intrinsic and extrinsic factors. TNF-α, an inflammatory cytokine, is one of the molecules for distrusting the CNS myelination (Titelbaum *et al.*, 2005, Kemanetzoglou & Andreadou, 2017, Andreadou *et al.*, 2013, Thomson *et al.*, 2008). TNF-α acts through its two distinctive receptors; TNFR1 and TNFR2. During apoptosis and necrosis, majorly TNFR1 receptor is active while during cell survival function mostly TNFR2 receptor is playing role (Sedger & McDermott, 2014, Probert, 2015). Reports are there which show that TNF-α is a well known trigger of apoptosis and necrosis of oligodendrocytes in both *in-vivo* and *in-vitro* conditions (Selmaj & Raine, 1988, Robbins *et al.*, 1987, Jurewicz *et al.*, 2005). With the support of those evidences, in this present study myelinating culture were treated with TNF-α in dose depended manner to find out effective dose of cytokine for inducing demyelination in culture. Results suggested that 20ng/mL TNF-α dose was ideal for induction of demyelination event rather than in inducing cell apoptosis. TNFR1 receptor was involved in the TNF-α induced demyelination in myelinating spinal cord culture. Decrease levels of MBP and myelin protein concentration, extracted from whole myelin, suggested establishment of demyelination condition in culture. In addition, result of ICC in dose depended TNF-α treated culture, also confirmed the establishment of *in-vitro* demyelination model. This *in-vitro* demyelination model was further used for the study of remyelination potential of BDNF and NGF neurotrophins. Neurotrophins are growth factors, essential for many important functions of the brain. NGF and BDNF are most important neurotrophins for cell survival and growth. NGF controls axonal receptivity (Chan *et al.*, 2004) to myelination and induce axon growth in myelinated portions of the CNS (Walsh *et al.*, 1999). BDNF plays a crucial role in myelin development (Xiao *et al.*, 2009), promotes peripheral myelination (Xiao *et al.*, 2013) and also acts as a neuroprotector in multiple sclerosis (Junhua, 2012). Here, in this study we assessed that during demyelination TNF-α reduced the levels of both these neurotrophins which created link between TNF-α and NGF /BDNF in CNS myelination regulation. To confirm the role of both neurotrophins' in regulation or remyelination, we studied the myelin proteins levels (MBP) in TNF-α alone treated group and TNF-α with neurotrophins (NGF

and BDNF) groups, and we found reduced level of MBP in TNF-α treated group, wereas the levels of MBP werenear normal in BDNF and NGF treated groups. Total myelin concentration, extracted from myelin sheath, was also higher in BDNF treated group compare to only TNF-α treated group. For more conformation regarding remyelination potential of BDNF and NGF, immunocytochemical analysis was carried out to assessmyelination in TNF-α alone group and TNF-α with BDNF and NGF treated groups; which proves that both the neurotrophins (BDNF and NGF) have potential to improve myelination in cytokine induced demyelination.

In overall conclusion, the present study confirmed the important role of BDNF and NGF in protection against the inflammation induced CNS demyelination by regulating the myelination and indicate their therapeutic potential to treat demyelination diseases such as Multiple sclerosis.