CHAPTER 4

To study the factors regulating MeCP2 phosphorylation

4.1 Introduction

During development, extrinsic and intrinsic stimuli create epigenetic marks (Delcuve et al., 2009). These epigenetic modifications thereby alter the chromatin structure and modulate the transcription capability (Olynik and Rastegar, 2012). It has been studied that MeCP2 is expressed at the level of histones and widely distributed across the genome. MeCP2 not only competes with the histones but also displaces them for chromatin binding sites (Ghosh et al., 2010; Nan et al., 1997). Studies in neurons have shown that diverse environmental stimuli affect MeCP2 function by differential phosphorylation (Zhou et al. 2006). MeCP2 S421 phosphorylation is induced in cortical neurons following synaptic transmission stimulation with glutamate, NMDA or GABAA-receptor antagonist bicuculline and neurotrophin treatment (Zhou et al., 2006). MeCP2 S421 phosphorylation is linked to cell growth signals by FGF in adult neuroprogenitor cells via the activation of aurora kinase (Li et al., 2014). Opposite to the dynamics of S421 phosphorylation, S80 is the most constitutively phosphorylated residue in resting neurons and undergoes dephosphorylation upon membrane depolarization (Tao et al., 2009). Moreover, MeCP2 S80 phosphorylation inhibits activation of certain genes (Tao et al., 2009). These studies highlight the relevance of stimuli-dependent MeCP2 phosphorylation at Ser80 and Ser421 sites.

During normal glial cell development in the CNS, both growth factors (GFs) and extracellular matrix (ECM) coexist in the cellular microenvironment, but the underlying mechanism by which they regulate glial cell behavior is poorly understood. The altered levels of GFs and ECM in the demyelinating lesions have been reported (Hughes et al., 2013). Two growth factors generally considered to play significant roles in oligodendrocyte development and myelination are PDGF and FGF2 (Armstrong et al., 1991; Bansal et al., 2003; Bansal et al., 2002; Fortin et al., 2005; Frost et al., 2009; Fruttiger et al., 1999; Guardiola-Diaz et al., 2012; Messersmith et al., 2000; Milner et al., 1997; Vora et al., 2011). Moreover, PDGF-A and FGF-2, are known to affect astroglial proliferation and maturation (Luo and Miller, 1999; Pringle et al., 1989; Reuss et al., 2003). Neurotrophins mainly NGF and BDNF are important in OLG migration and proliferation in CNS (Tirassa et al., 2003; Van't Veer et al., 2009). BDNF and NGF has

an anti-proliferative effect when the astrocytes are also exposed to a mitogen i.e they play a role in differentiation (Cragnolini et al., 2009).

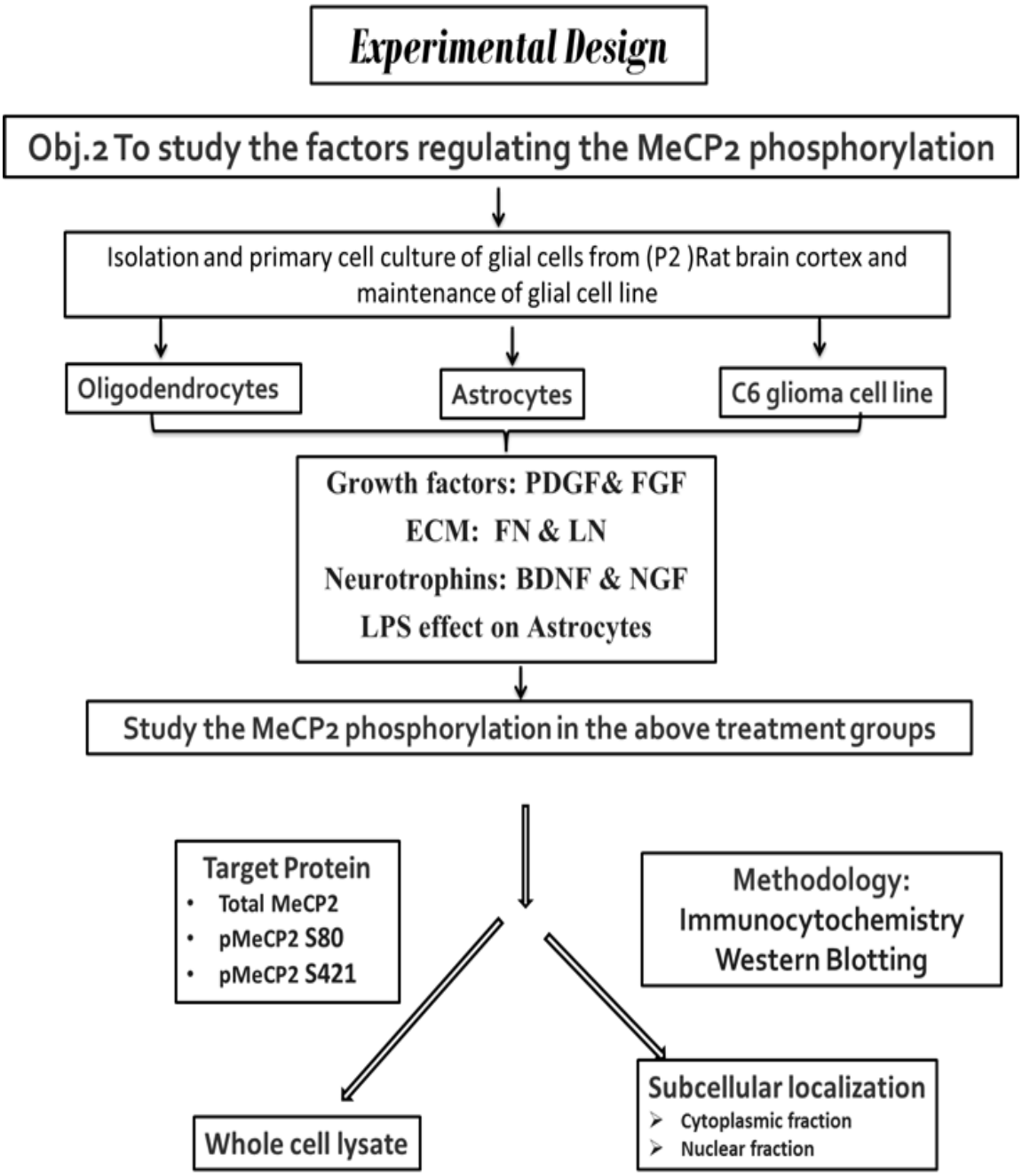
Not only GFs but ECMs like FN and LN also regulate the OLG behavior (ffrench-Constant et al., 1994; Milner et al., 1997; Milner et al., 1996; Tripathi, 2017). LN and FN also have significant effects on astrocyte growth (Johnson et al., 2015). Interestingly, in response to LPS stimulation astrocytes become reactive and are involved in shaping the innate immune response by proper scar formation, promoting BBB repair thereby supporting neuronal survival (Farina et al., 2007). Moreover, when MeCP2 deficient astrocytes are stimulated by LPS, the levels of TNF-α, IL-1β, and IL-6 were dramatically decreased compared to the wild type (Maezawa et al., 2009). Therefore, it becomes important to study the effect of LPS on MeCP2 phosphorylation. The role of each of these growth factors and ECM is briefly described in Table 4.1

So far, no study has been carried out to demonstrate the effects of these developmental stimuli on MeCP2 phosphorylation in glial cells. Hence, the focus of the present study was to evaluate individual effects of PDGF-A, FGF, FN, LN, NGF and BDNF on the MeCP2 phosphorylation at pS80 and pS421 sites in glial cells (OLGs and astrocytes) of CNS. Stimuli regulated expression of pMeCP2s (pS80 & pS421) were analyzed in growth factor treated in vitro primary cultures of rat glial cells and their corresponding cell lines. Further, the sub-cellular localization of phosphorylated MeCP2s was also assessed after the treatment with various growth factors in the cultures of glial cells.

Table 4.1 Factors affecting glial cell development

Factors	CNS Glial cell type	Role/Study	References
Growth factors PDGF & FGF	Oligodendrocytes	• Migration • Proliferation	Frost et al., 2009 Vora et al., 2011 Hebe et al., 2012 O'meara et al., 2011
	Astrocytes	• Migration • Proliferation	Luo & Miller, 1999
Extracellular matrix FN & LN	Oligodendrocytes	• Migration & Proliferation(FN) • Differentiation and myelination (LN)	O'Meara et al., 2011
	Astrocytes	• Proliferation	Johnson et al., 2015
Neurotrophins BDNF & NGF	Oligodendrocytes	• OPC proliferation & development via ERK pathway • Differentiation and Myelination	Van't et al., 2009 VonDran et al., 2011 Tirassa et al., 2003
	Astrocytes	• Maturation	Ortega & Alcántara,2010 Cragnolini et al., 2009
LPS	Astrocytes	• Inflammation	Farina et al., 2007

4.2 Plan of Work



4.3 Results

4.3.1 MeCP2 is phosphorylated at S80 in response to various growth factors and ECM in immature OLGs

In the present study, the individual effects of PDGF-A, FGF, BDNF, NGF, FN, and LN on the MeCP2 phosphorylation at pS80 in OLGs were investigated. PLL was used as control matrix. The data indicates that immature OLGs exposed to LN resulted in enhanced expression of pS80MeCP2 (1.71 fold, **$p < 0.01$) compared to other treatment groups [Fig-4.1 (A, B)]. Morphological analysis of OLGs showed that LN significantly increased the differentiation of immature OLGs to mature OLGs [Fig-4.1 (C)]. There was no change in the expression level of pS80MeCP2 ($p > 0.05$) in neurotrophins treated OLGs [Fig-4.1 (D, E)]. Thus, these observations indicate the importance of ECM more than GFs in the regulation of MeCP2 phosphorylation at S80 site. Hence, the effects of FN and LN on mature OLGs were examined. Following standard protocols, OLGs were allowed to mature (described in Materials and Methods- chapter 2 sections).

4.3.2 Laminin Differentially Regulates the Expression of pS80MeCP2 in Immature and Mature OLGs

The data also confirmed that LN enhanced the expression of MBP (1.80 fold, ***$p < 0.001$) in mature OLGs. Maturation of OLGs was confirmed by the MBP expression study using ICC and Western Blotting [Fig-4.2 (A, B)]. Earlier, it has been shown that MeCP2 regulates MBP expression in OLGs (Sharma et al. 2015). The mature OLGs were exposed to FN and LN. PLL was used as a control matrix. Western blot analysis showed significant decrease in pS80MeCP2 (0.5 fold, **$p < 0.01$) expression levels in the mature OLGs exposed to LN compared to those exposed to FN and PLL (control) [Fig-4.2 (C, D)]. These observations were further confirmed by pS80MeCP2 nuclear localization studies using confocal microscopy imaging. The data showed significant decrease in the LN-dependent nuclear pS80MeCP2 expression and co-localization as compared to the PLL control [Fig-4.2 (E)]. Data from the present study shows that LN

regulates MeCP2 phosphorylation at S80 site and MBP, both. Hence, pS80MeCP2 may be a link between LN-dependent MBP expressions.

4.3.3 Growth factors and ECM had no effect on MeCP2 phosphorylation in astrocytes

Next, the study investigated the individual effects of PDGF-A, FGF, BDNF, NGF, FN, and LN on the MeCP2 phosphorylation at S80 and S421 in astrocytes. It was revealed that there was no significant change ($p > 0.05$) in the levels of pS80MeCP2 and pS421MeCP2 when astrocytes were treated with ECMs like FN and LN [Fig-4.3 (A, B, C)]. Moreover, the PDGF-A and FGF treatment in astrocytes also did not affect the levels of pS80MeCP2 and pS421MeCP2 ($p > 0.05$) [Fig-4.3 (D, E, F)]. The data suggests that MeCP2 phosphorylation in astrocytes is not affected by ECM and growth factor treatment.

4.3.4 BDNF significantly increase the pS421MeCP2 phosphorylation in astrocytes

Next, the study showed that BDNF could significantly increase the MeCP2 phosphorylation at S421 (Fig-4.4) whereas PDGF, FGF, LN and FN each had no effect on MeCP2 S80 and S421 phosphorylation (Fig-4.3). The cortical astrocytes were treated with 50ng/ml BDNF and 50ng/ml NGF for 1hour after 24hours of serum starving. The results indicate a significant increase in the pS421MeCP2 (3.4 fold, $^{**}p < 0.01$) expression in BDNF treated group whereas there was no change in the pS80MeCP2 expression ($p > 0.05$) in the BDNF and NGF treated groups compare to the controls [Fig-.4.4 (A-C)]. Subsequently, the localization of neurotrophin induced pS421MeCP2 expression in cytoplasmic and nuclear fractions of control and neurotrophin treated astrocytes were also studied. MeCP2 and pS80MeCP2 were concentrated in the nucleus in control and neurotrophin treated samples [Fig-.4.4 (D, G)], whereas pS421MeCP2 was localized in the cytoplasm as well as nucleus [Fig-.4.4 (D-F)]. Moreover, in BDNF

treated group pS421MeCP2 expressed in cytoplasm at a relatively low level than in the nucleus [Fig-4.4 (D)]. These results demonstrate that, among the many growth factors and ECM tested; only BDNF trigger the phosphorylation of MeCP2 at S421 which suggest that BDNF signaling may regulate MeCP2 function.To confirm the intracellular localization of pS421MeCP2, immunofluorescentdoublestaining was performed with DAPI in the astrocytes treated with BDNF. It was observed that pS421MeCP2 in BDNF treated astrocytes [Fig-4.4 (H-b, d, f)] was completely co-localized with DAPI, while pS421MeCP2 in control [Fig-4.4 (H-a, c, e)] was found to be mainly localized into the cytoplasm, consistent with thefindings of western blot analysis [Fig-.4.4 (D, G)]. These results demonstrated that the intracellular localizations of MeCP2 and the pMeCP2s are distinct when treated with BDNF.

4.3.5 Neurotrophin stimulation in C6 glioma cell line increases the MeCP2 phosphorylation at S80 and S421

Subsequently, the effect of neurotrophins on C6 gioma cell line which is an astrocytic cell line was also studied. The neurotrophinstreatments with BDNF and NGF significantly increased the levels of pS421MeCP2 levels (Fig-.4.5). The results indicate a significant increase in the pS421MeCP2 (**$p < 0.01$ for BDNF; *$p < 0.05$ for NGF) expression in BDNF and NGF treated groups [Fig-.4.5 (A, B)]. Unlike astrocytes C6 glioma cells showed increase in pS80MeCP2 expression (**$p < 0.01$ for BDNF; *$p < 0.05$ for NGF) in the same set of groups [Fig-.4.5 (A, B)]. These results demonstrate that, there is a differential regulation of MeCP2 phosphorylation by neurotrophins in C6 glioma cells suggesting that neurotrophin signaling may regulate MeCP2 function. Moreover, the localization of pS421MeCP2 phosphorylation in cytoplasmic and nuclear fractions of C6 glioma cells was examined [Fig-.4.5 (D-F)]. It was observed that the pS421MeCP2 was localized into the nucleus after the treatment with BDNF and NGF. Thereby, stating the importance of pS421MeCP2 in regulating glioma cells via neurotrophins.

4.3.6 LPS promoted the pS421 phosphorylation of MeCP2 and its intracellular localization in Astrocytes:

Firstly the effect of LPS on cortical primary astrocytes was confirmed. LPS (100ng/ml) treatment with astrocytes for 24 hours leads to up regulation of GFAP expression which is a marker for reactive astrocytes [Fig.-4.6(A)]. The western blot data clearly indicates that when the cortical astrocytes are treated with LPS induces the GFAP level (1.69 fold, $^{**}p < 0.01$) thereby making the astrocytes reactive [Fig.-4.6 (B, C)]. Next, the effect of LPS on MeCP2 phosphorylation was studied. There was a significant increase in pS421 phosphorylation of MeCP2 (2.5 fold, $^{***}p < 0.001$) when astrocytes are treated with LPS [Fig-4.7 (A, B)]. However, there was no change in the pS80MeCP2 level ($p > 0.05$) in the control and LPS treated astrocytes [Fig-4.7 (A, C)]. Interestingly, when the localization of MeCP2 phosphorylation was checked in the cytoplasmic and nuclear fraction increase in pS421MeCP2 level in cytoplasm was observed as compared to the nucleus [Fig-4.7 (D)].There was no change in the localization as well as expression of pS80MeCP2 and pS80MeCP2 was seen to be localized in the nucleus [Fig-4.7 (D,G)]. However, there was increase in pS421MeCP2 level in LPS treated group compared to control [Fig-4.7 (D, E, F)]. To confirm the intracellular localization of MeCP2 and the pS421MeCP2, immunofluorescentdoublestaining was performed with DAPI in the astrocytes treated with LPS. It was observed that the pS421MeCP2 in LPS treated astrocytes was found to be concentrated into the cytoplasm [Fig. 4.7 (H)], consistent with the findings of western blot analysis [Fig-.4.7 (D)].

4.4 Discussion

In recent years, it has become clear that phosphate groups are important in controlling protein function. Protein phosphorylation is involved in diverse biological activities including transcriptional control, DNA repair, mRNA splicing, signal transduction, and protein translocation (Bedford and Clarke, 2009; Boisvert et al., 2003). In this chapter it was confirmed that MeCP2, which is a key contributor of glial cell function, its phosphorylation at S80 and S421 sites is modulated by different developmental stimuli

which further leads to its subcellular localization. Firstly the effect of different factors on MeCP2 phosphorylation at S80 site in OLGs was analyzed. OLG shows different phases during development like early PDGF receptor alpha (PDGFRα)-positive OPCs, followed by O4-positive immature OLGs, and lastly differentiated MBP-positive mature OLGs (Yang et al., 2011). OLG development is tightly regulated by developmental growth factors and ECM (Milner et al., 1998). OPCs continue to proliferate but fail to differentiate in the presence of FGF, PDGF, NGF and BDNF. Removal of these GFs is essential for the proper differentiation of the OPCs to OLGs (Noble et al. 1988; Raff et al. 1988). Reports have shown that OPC migration also occurs in the absence of GFs, but OPCs must be plated on a permissive substratum (Frost et al., 1996; Fruttiger et al., 1999; Kakita and Goldman, 1999; Tripathi, 2017). FN promotes OLG migration and proliferation, but reduces myelin-like membrane formation (Baron et al., 2005; Frost et al., 1999; Maier et al., 2005; Šišková et al., 2006; Tripathi, 2017). LN is a well characterized ECM for OLG survival (Baron et al., 2003; Colognato et al., 2002) and myelination (Buttery, 1999; Relvas et al., 2001; Šišková et al., 2006). The results show that unlike other growth factors, ECM i.e. FN and LN significantly increased the phosphorylated pS80MeCP2 expression in immature OLGs (Fig-4.1. & 4.2). ECM plays a very critical role not only during normal myelination but also during remyelination. OPC differentiation is dependent on the external regulation. The extracellular matrix is extensively altered during CNS injury (Back et al., 2005; Gutowski et al., 1999; Sobel and Mitchell, 1989; van Horssen et al., 2006). LN is present on the surface of neurons, and OLGs contact neuronal LN through α6β1 integrin (O'Meara et al., 2011; Simons and Trajkovic, 2006). Hence when the effect of these ECMs on mature OLGswere checked, a substantial decreasewas observedin pS80MeCP2 expression levels in the mature OLGs exposed to LN compared to those exposed to FN and PLL [Fig-.4.2 (C, D)]. The data also confirmed that LN enhanced the expression of MBP in mature OLGs [Fig-.4.2 (A)]. Earlier studies demonstrated that MeCP2 regulates MBP expression in OLGs (Sharma et al., 2015). Data from the present study shows that LN regulates MeCP2 phosphorylation at S80 site and MBP, both. Hence, these data suggest that pS80MeCP2 may participate in the regulation of MBP during OLG differentiation.

Next, the effect of these growth factors on MeCP2 S80 and S421 phosphorylation in astrocytes was examined. The study clearly shows that there is no change in the MeCP2 phosphorylation at S80 and S421 when astrocytes are treated with ECM and growth factors (Fig-4.3). Whereas there is a significant increase in pS421MeCP2 levels by neurotrophins like BDNF but the pS80MeCP2 level was not affected(Fig-4.4).BDNF plays a development dependent modulatory role in serotonergic neurons and glial cells;BDNF is an essential factor for the development of a number of glial properties (Djalali et al., 2005). While NGF during development participate in regulating the transition from a dividing to a differentiating population of cells (Cragnolini et al., 2009). Previously it was reported that neurotransmission stimulation with glutamate, NMDA or GABAA-receptor antagonist bicuculline and neurotrophin treatment aresufficient to induce MeCP2 S421 phosphorylation in cortical neurons (Zhou et al., 2006). On the contrary, MeCP2 at S80 sites undergoes dephosphorylation upon membrane depolarization (Tao et al., 2009). Consistent with the previous reports, the current study revealed the increased expression of pS421MeCP2 in the BDNF treated group of astrocytes compared to the control groups on the contrary there was no change in the pS80MeCP2 expression in the BDNF and NGF treated groups compare to the controls (Fig-4.4). Mutation of the S80 phosphorylation site reduces MeCP2 association with chromatin at several euchromatic gene promoters thereby alters transcription of several genes that are potentially important for neuronal function (Tao et al., 2009). In addition to this, Gonzales et al. suggested that phosphorylation of MeCP2 at S80 may influence the association of MeCP2 to transcription repressor Sin3A, heterochromatin protein 1 (HP1), cohesin complex member SMC3, and RNA binding protein YB1 (Gonzales et al., 2012).Whereas there is an opposing role of pS421MeCP2 in neurons. The gene activation mediated by MeCP2 is through the phosphorylation of MeCP2 at S421 which may be permissive for changes in transcriptional regulation rather than for inhibition of gene activation (Li et al., 2014). Moreover, there are reports suggesting that these sites are being regulated by external stimuli and many have reported different stimuli affecting the phosphorylation of MeCP2 at S80 and S421 sites there by changing the function of MeCP2 and this in turn which will affect the gene expression (Refer Chapter 1-Introduction). Two of the studies reported that in the absence of membrane depolarization, MeCP2 is bound to Bdnf promoter IV and prevents its transcription,

while it is phosphorylated and released from that promoter after KCl-induced neuronal stimulation, resulting in the activation of Bdnf transcription (Ballas et al., 2005; Chen et al., 2003). Apart from this it is also reported that BDNF secreted by neuronal activity can trigger MeCP2 phosphorylation and its release from Bdnf promoter, the resulting Bdnf transcription was proposed to underlie the well-known positive feedback loop of BDNF induced BDNF synthesis (Chen et al., 2003; Zhou et al., 2006). On this basis, the enhanced phosphorylation of MeCP2 at S421 after BDNF treatment may affect the gene transcription of Bdnf gene in astrocytes. Moreover, it is also reported that S100b was increased in primary cultures of pure astrocytes by exogenous BDNF (Djalali et al., 2005). Further, it is shown that MeCP2 regulates the expression of astroglial marker transcripts, including GFAP and S100β in cultured astrocytes (Okabe et al., 2012). In conjunction with these reports, it can be speculated that increased pS421MeCP2 may regulate the expression of S100β stimulated by BDNF. However, the underlying molecular mechanisms need to be investigated.

Next the effect of LPS on MeCP2 phosphorylation in astrocytes was investigated.An important astrocytic function after activation by lipopolysaccharides (LPS) is to initiate and regulate immune responses through the release of proinflammatory cytokines to produce a protective scar around the injury (Farina et al., 2007; Martin-Subero et al., 2016). In the present study innovatively revealed that pS421MeCP2 was significantly upregulated and also found to be localized in cytoplasm (Fig. 4.7). MeCP2 being a transcriptional regulator should be localized into nucleus but in this study when astrocytes were treated with LPS it was localized into the cytoplasm suggesting its role in cytoplasm.To date, most studies considered MeCP2 and phosphorylated MeCP2 as nucleoproteins (Cohen et al., 2011; Gonzales et al., 2012; Mnatzakanian et al., 2004), although a previous research study showed that MeCP2 can be repositioned from the cytoplasm to the nucleus upon neuronal differentiation by introducing de-phosphorylation on serine sites (Miyake and Nagai, 2007). In accordance with this finding, it is revealed that pS421MeCP2 is mainly localized in the cytoplasm, whereas total MeCP2 was found in the nucleus in both control and LPS treated astrocytes [Fig-4.7 (D-G)] suggesting a distinctive role of pS421MeCP2 in the cytoplasmic-related events. Interestingly, Maezawa et al revealed that in response to LPS stimulation, MeCP2-

deficient astrocytes released fewer cytokines such as IL-1β and IL-6 (Maezawa et al., 2009). Therefore, the data suggests that increased cytoplasmic pS421MeCP2 in response to LPS stimulation might play an important role in the reactive astrocytes and regulate immune responses during injury in CNS.

Fig 4.1

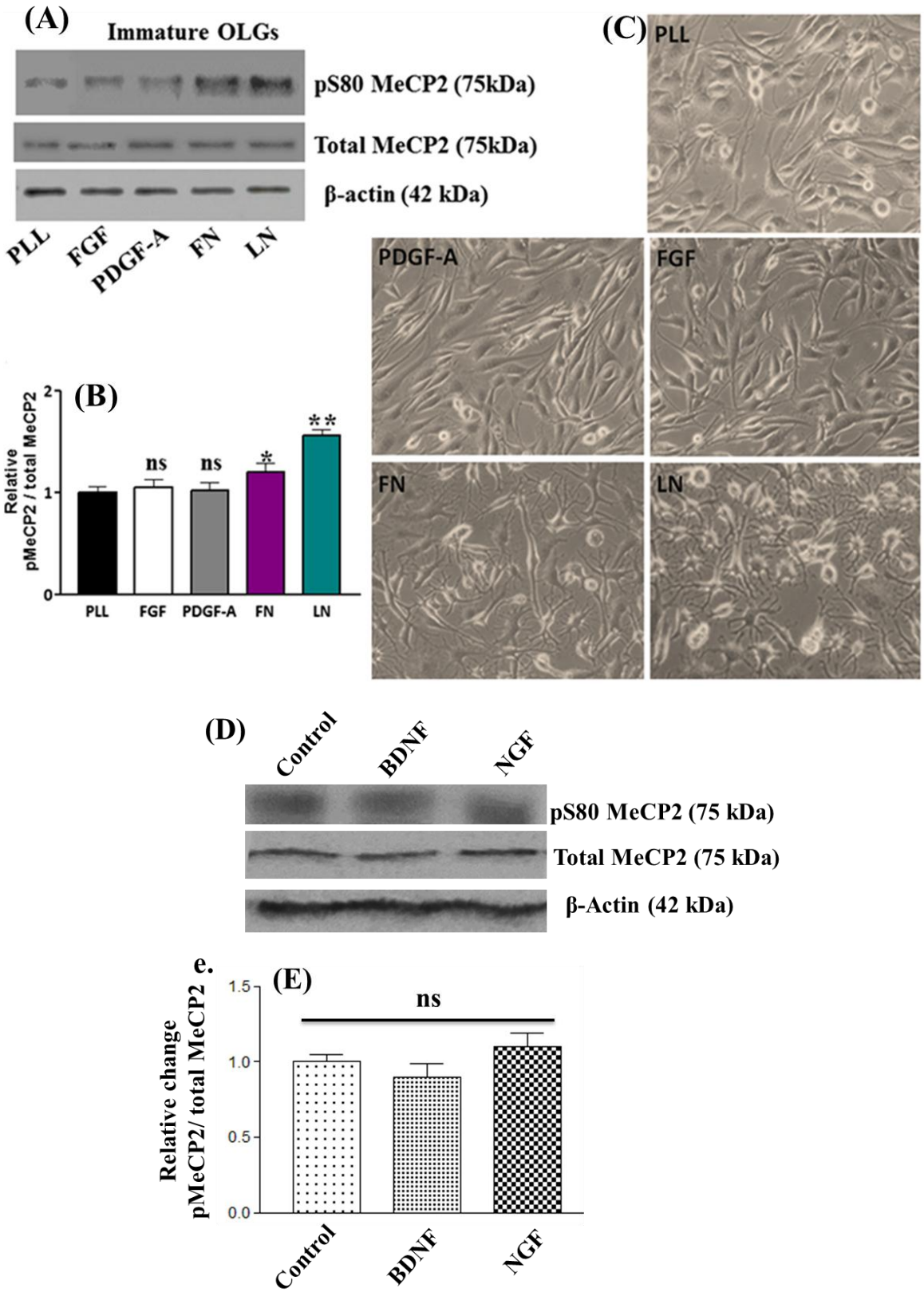

(A)
Immature OLGs
pS80 MeCP2 (75kDa)
Total MeCP2 (75kDa)
β-actin (42 kDa)
PLL
FGF
PDGF-A
FN
LN
(B)
Relative pMeCP2 / total MeCP2
ns
ns
*
**
(C)
PLL
PDGF-A
FGF
FN
LN
(D)
Control
BDNF
NGF
pS80 MeCP2 (75 kDa)
Total MeCP2 (75 kDa)
β-Actin (42 kDa)
e.
(E)
Relative change pMeCP2/ total MeCP2
ns

Fig. 4.1 MeCP2 S80 phosphorylation by growth factors and ECMin immature OLGs*(A) Representative Western blot for pS80MeCP2 and total MeCP2 in immature OLGs exposed to FGF, PDGF-A, FN, and LN for 1 hr. (B) Graph shows increased levels of pS80MeCP2 in LN-treated group. Values represent mean ± SEM. (C) Representative phase contrast images of immature OLGs exposed to FGF, PDGF-A, FN, and LN for 24 hr for morphological analysis. (D) Representative Western blot for pS80MeCP2 and total MeCP2 in immature OLGs exposed to BDNF and NGF for 1 hr. (E) Graph shows BDNF and NGF had no effect on pS80MeCP2 levels.Three different cell preparations were analyzed three times N= 3. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using one-way ANOVA parameter where appropriate (*P < 0.05, **P < 0.01, ***P <*

Fig 4.2

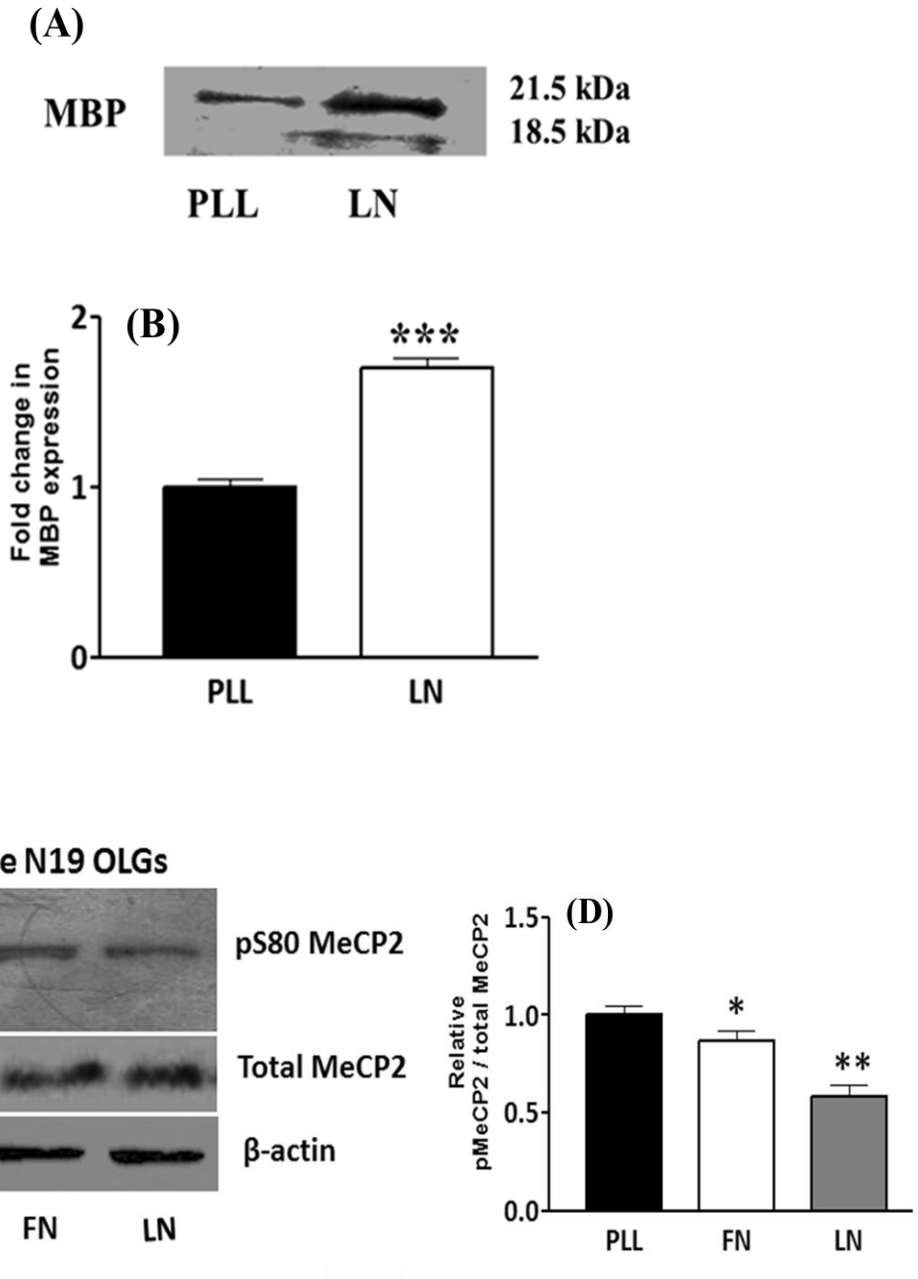

(A)
MBP
21.5 kDa
18.5 kDa
PLL
LN
(B)
Fold change in MBP expression
2
1
0
***
PLL
LN
(C) Mature N19 OLGs
pS80 MeCP2
Total MeCP2
β-actin
PLL
FN
LN
(D)
Relative pMeCP2 / total MeCP2
1.5
1.0
0.5
0.0
*
**
PLL
FN
LN

Fig 4.2 (E)

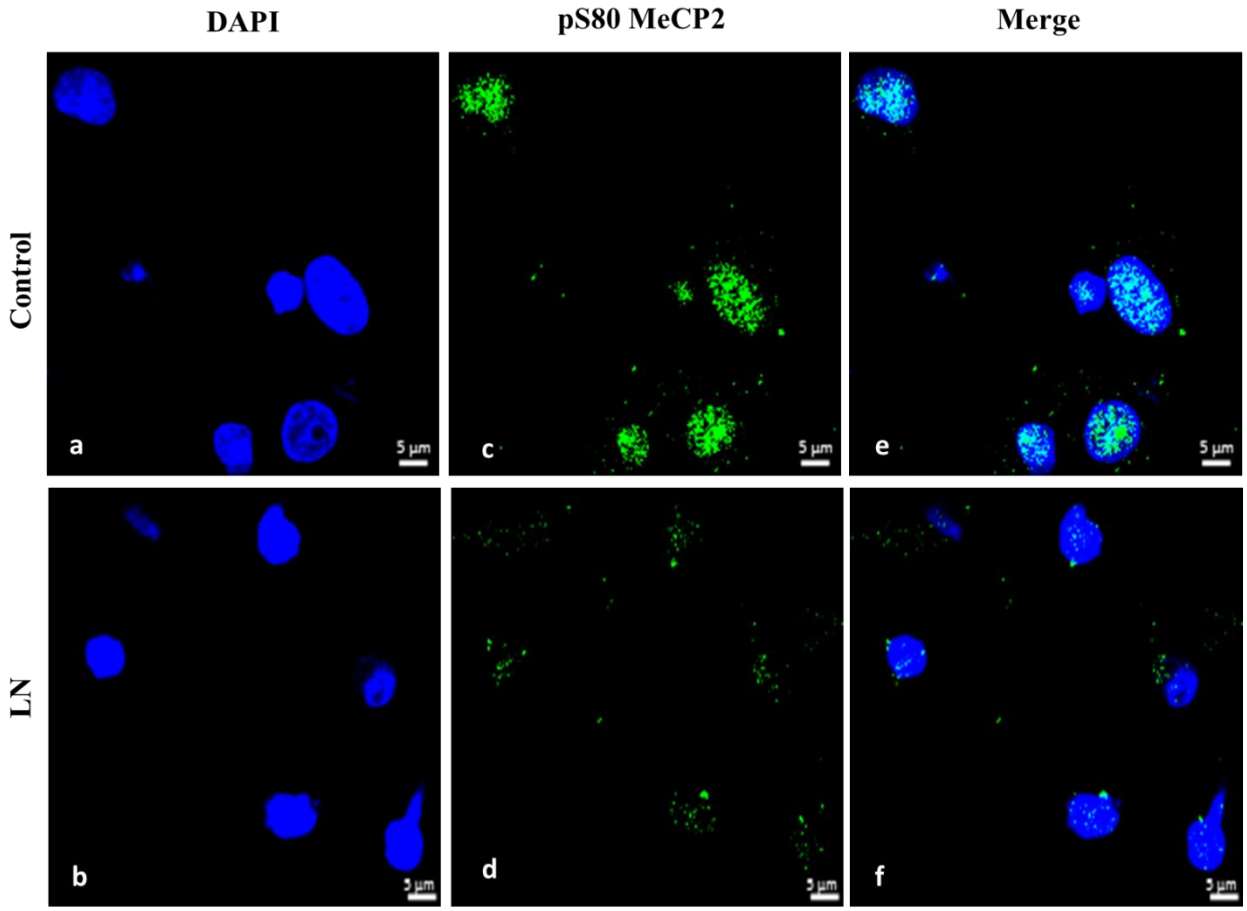


Fig 4.2 Laminin Differentially Regulates the Expression of pS80MeCP2 in Immature and Mature OLGs *(A) Representative Western blot for MBP in OLGs exposed to PLL control and LN. (B) Graph shows increased levels of MBP in LN-treated group. (C) Representative Western blot for pS80MeCP2 in immature and mature OLGs. (D)Graph shows increased levels of pS80MeCP2 in mature OLGs.Three different cell preparations were analyzed three times N= 3. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's t test or one-way ANOVA parameter where appropriate (*P < 0.05, **P < 0.01, ***P < 0.001). (E) Representative immunostaining images showing pS80MeCP2 expression in OLGs (green; c,d). The nucleus was stained with DAPI (blue; a,b). Colocalization of pS80MeCP2 and DAPI shown in merge images (e,f). Scale bar= 5µm.*

Fig 4.3

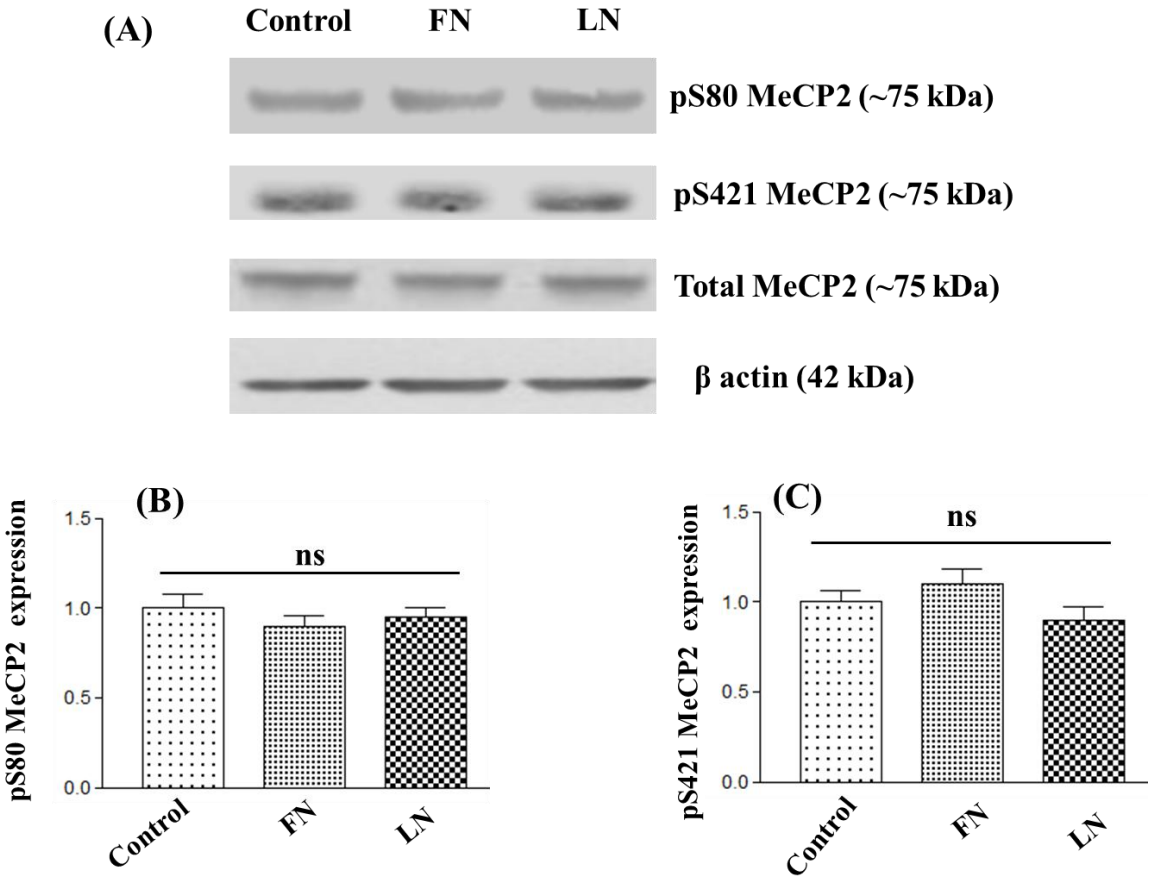

(A)
Control
FN
LN
pS80 MeCP2 (~75 kDa)
pS421 MeCP2 (~75 kDa)
Total MeCP2 (~75 kDa)
β actin (42 kDa)
(B)
pS80 MeCP2 expression
1.5
1.0
0.5
0.0
ns
Control
FN
LN
(C)
pS421 MeCP2 expression
1.5
1.0
0.5
0.0
ns
Control
FN
LN

Fig 4.3

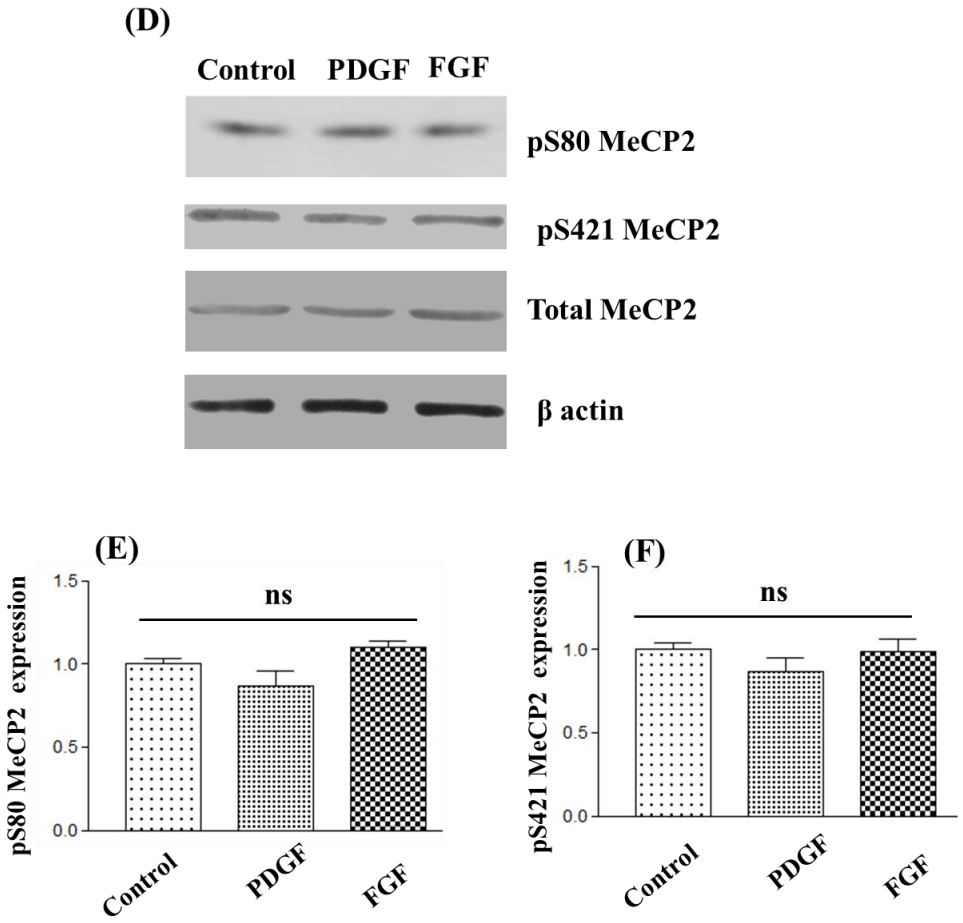


Fig.4.3 MeCP2 phosphorylation by growth factors and ECM in Astrocytes: *(A)Representative Western blot for pS80MeCP2, pS421MeCP2 and total MeCP2 in cortical astrocytes exposed to FN and LN for 1 h after 24 hours of serum starving. (B) Graph shows FN and LN had no effect on pS80MeCP2. (C) Graph shows FN and LN had no effect on pS421MeCP2 phosphorylation. (D)Representative Western blot for pS80MeCP2, p S421 MeCP2 and total MeCP2 in cortical astrocytes exposed to FGF and PDGF-A for 1 h after 24 hours of serum starving. (E) Graph shows no significant difference in the levels of pS80MeCP2 in the FGF and PDGF-A treated groups compared to control. (F) Graph shows no significant difference in the levels of pS421MeCP2 in the FGF and PDGF-A treated groups compared to control.Three different cell preparations were analyzed three times N= 3. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's t test or one-way ANOVA parameter where appropriate (*P < 0.05, **P < 0.01, ***P < 0.001)*

Fig 4.4

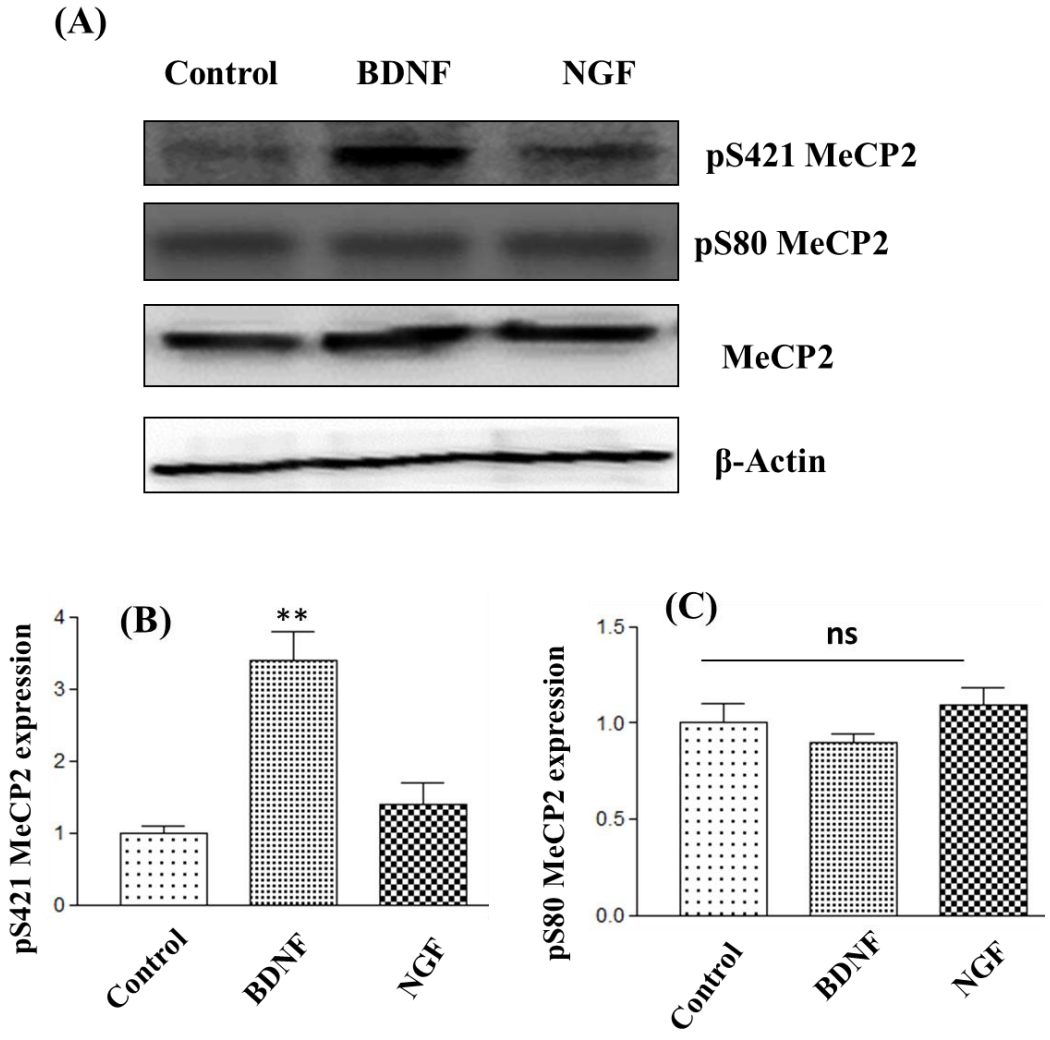

(A)
Control
BDNF
NGF
pS421 MeCP2
pS80 MeCP2
MeCP2
β-Actin
(B)
**
pS421 MeCP2 expression
0
1
2
3
4
Control
BDNF
NGF
(C)
ns
pS80 MeCP2 expression
0.0
0.5
1.0
1.5
Control
BDNF
NGF

Fig 4.4

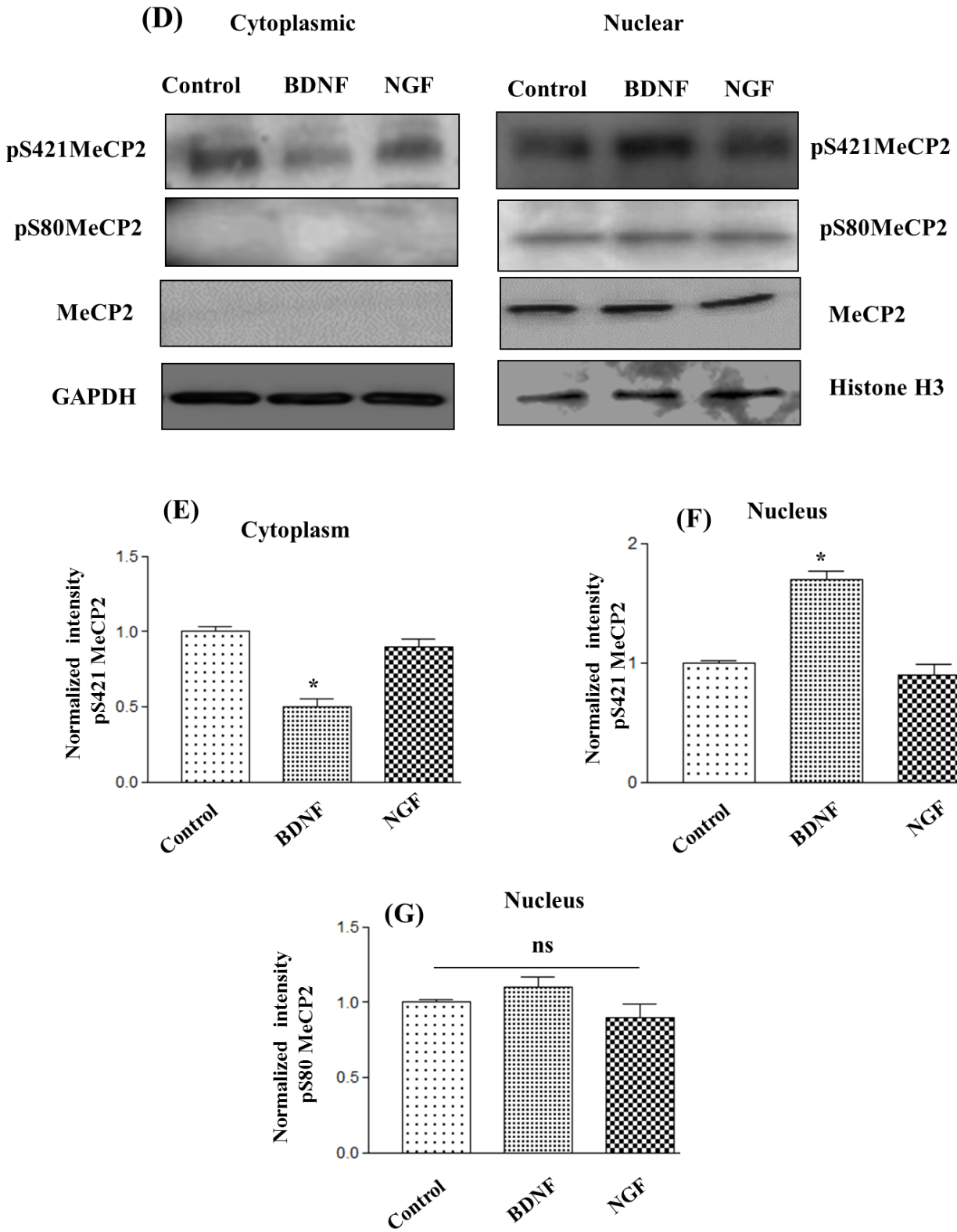

(D)
Cytoplasmic
Nuclear
Control
BDNF
NGF
Control
BDNF
NGF
pS421MeCP2
pS80MeCP2
MeCP2
GAPDH
pS421MeCP2
pS80MeCP2
MeCP2
Histone H3
(E)
Cytoplasm
Normalized intensity pS421 MeCP2
1.5
1.0
0.5
0.0
*
Control
BDNF
NGF
(F)
Nucleus
Normalized intensity pS421 MeCP2
2
1
0
*
Control
BDNF
NGF
(G)
Nucleus
ns
Normalized intensity pS80 MeCP2
1.5
1.0
0.5
0.0
Control
BDNF
NGF

Fig 4.4 (H)

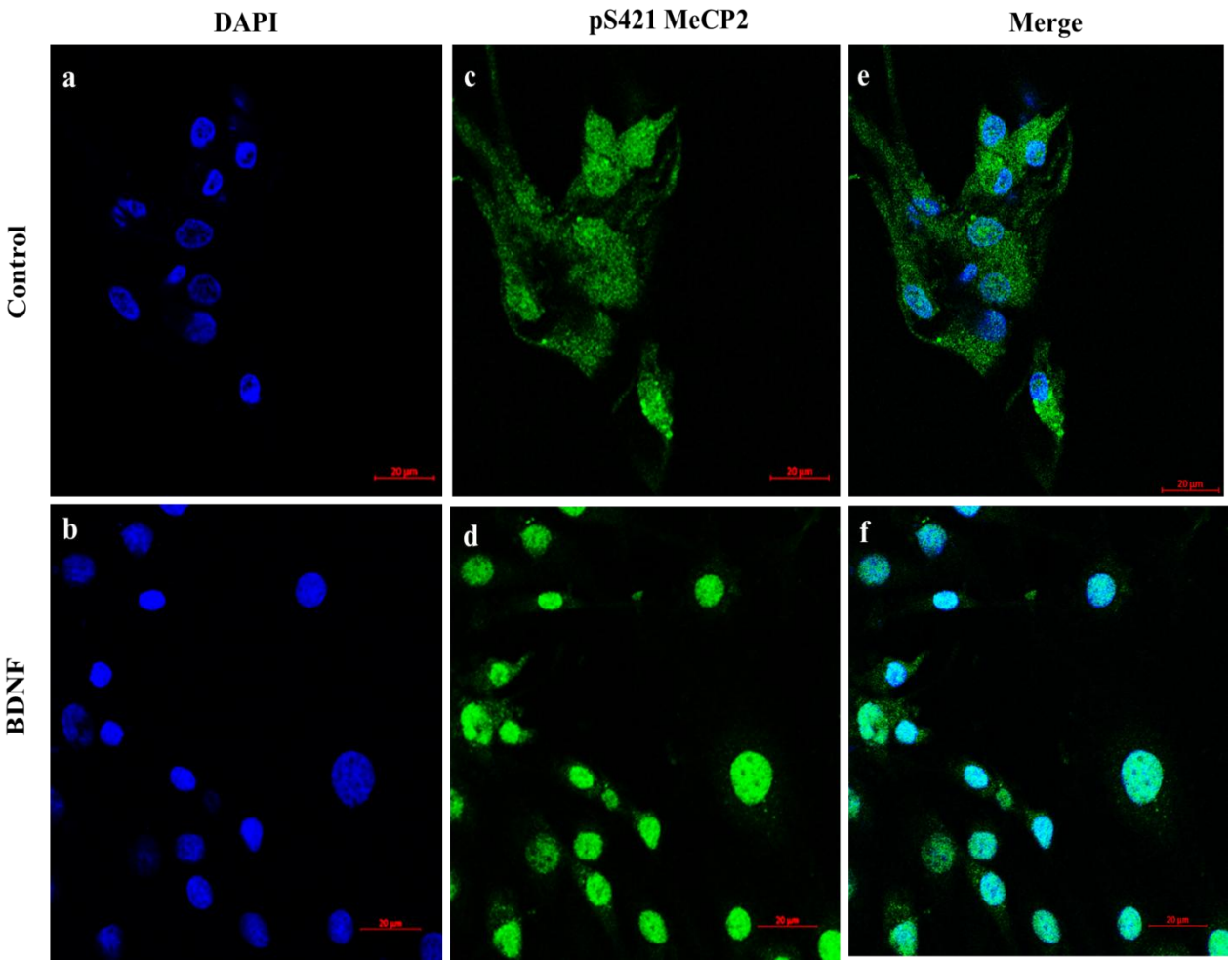


Fig. 4.4 MeCP2 phosphorylation by neurotrophins in Astrocytes*: (A) Representative Western blot for p S80 MeCP2, p S421 MeCP2 and total MeCP2 in cortical astrocytes exposed to NGF and BDNF for 1hr after 24hrs of serum starving. (B) Graph shows increased levels of pS421MeCP2 in BDNF treated group compared to control. (C) Graph shows no significant change in the levels of pS80MeCP2 in BDNF treated group. (D) Western blot analysis was applied to the cytoplasmic and nuclear proteins of the control and neurotrophins treated groups. MeCP2 and pS80MeCP2 were mainly found in the nuclear fraction, while pS421MeCP2 was found to be concentrated in cytoplasm than nucleus. GAPDH was used as loading controls for the cytoplasmic proteins, and H3 for the nuclear proteins. (E-G) After densitometric analysis, pS421MeCP2 and pS80MeCP2 data were normalized to the loading controls of cytoplasmic and nuclear fractions. (E,F) Graph shows pS421MeCP2 levels are high in nucleus in BDNF treated group as compared to control (G) Graph shows no significant difference in the pS80MeCP2 levels in the neurotrophins treated groups (H)Representative immunostaining images showing pS421MeCP2 expression in astrocytes (green; c,d). The nucleus was stained with DAPI (blue; a,b). Colocalization of pS80MeCP2 and DAPI shown in merge images (e,f).Three different cell preparations were analyzed three times N= 3. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's t test or one-way ANOVA parameter where appropriate (*P < 0.05, **P < 0.01, ***P < 0.001)*

Fig 4.5

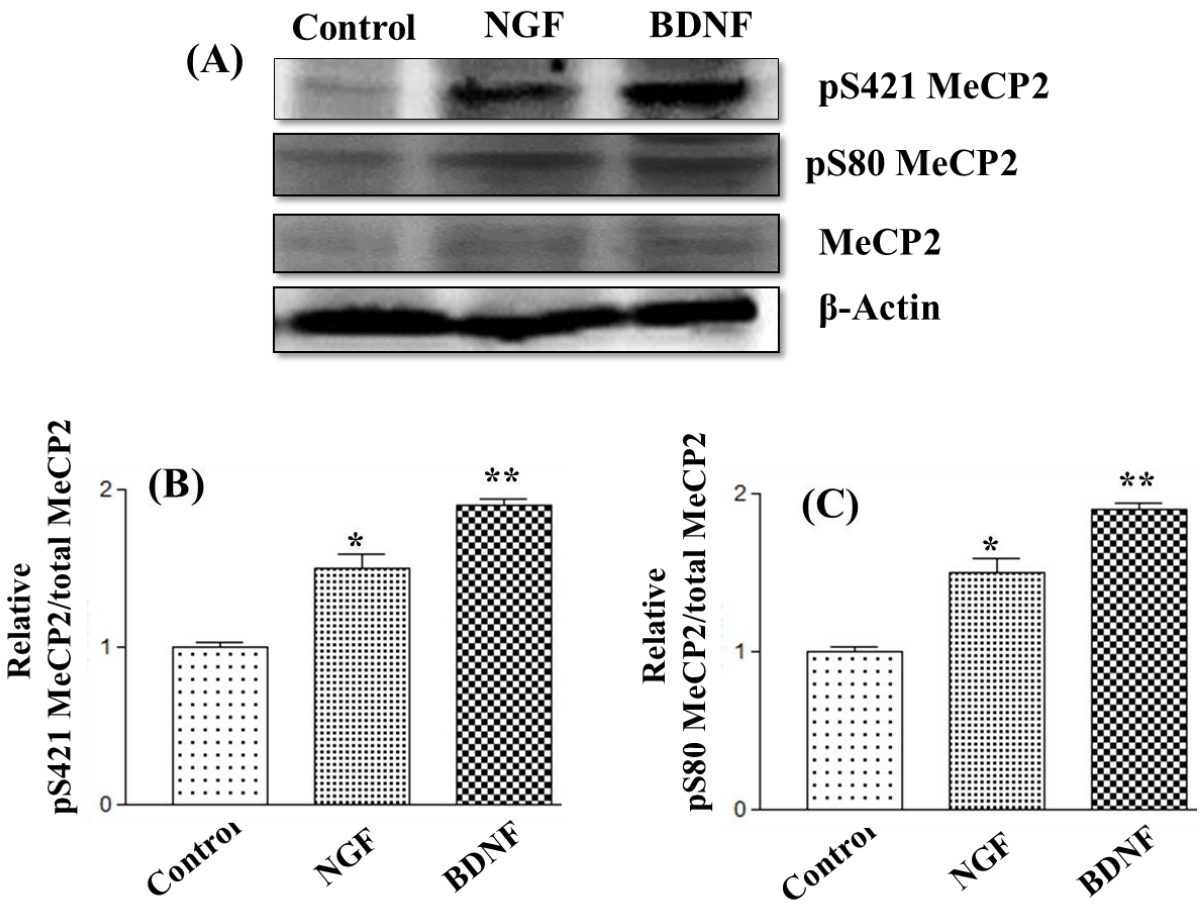

(A)
Control
NGF
BDNF
pS421 MeCP2
pS80 MeCP2
MeCP2
β-Actin
(B)
Relative pS421 MeCP2/total MeCP2
0
1
2
*
**
Control
NGF
BDNF
(C)
Relative pS80 MeCP2/total MeCP2
0
1
2
*
**
Control
NGF
BDNF

Fig 4.5

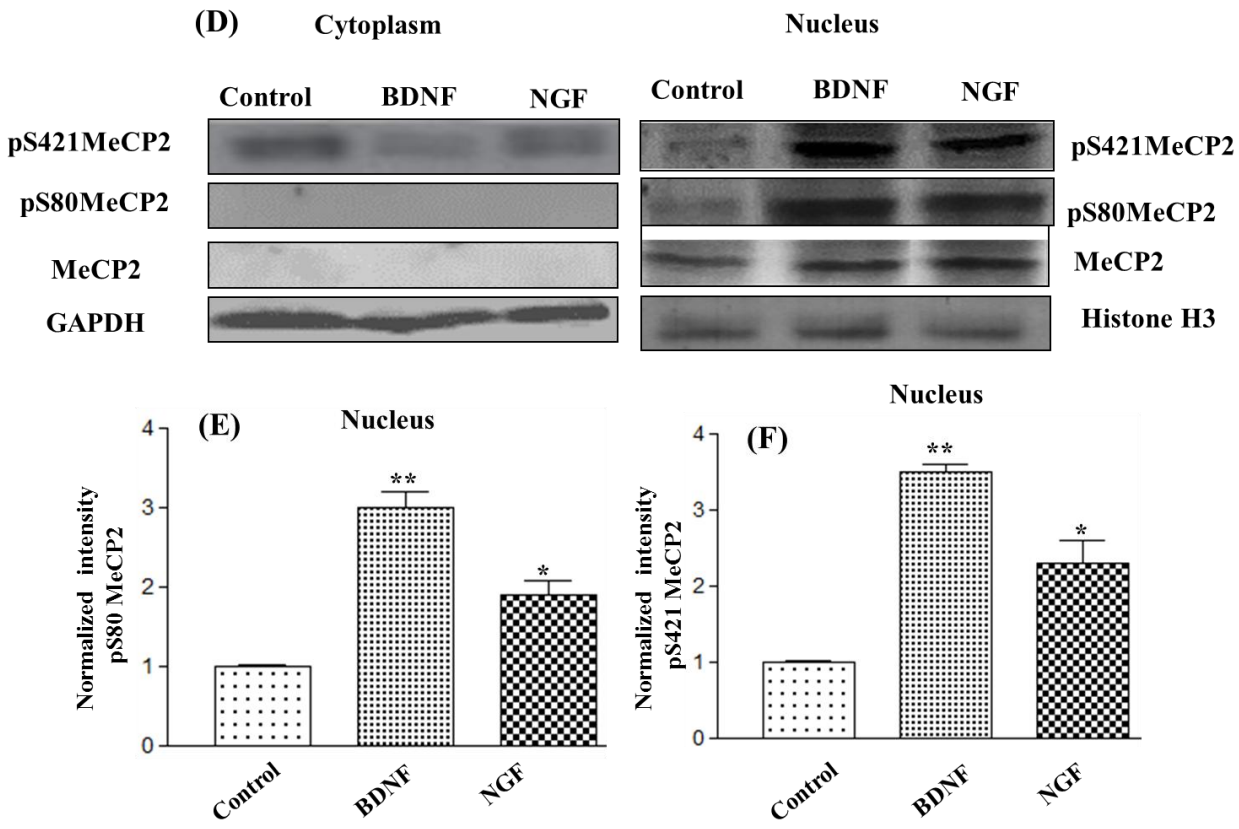


Fig. 4.5 MeCP2 phosphorylation by neurotrophins in C6 glioma*: (A) Representative Western blot for pS80MeCP2, pS421MeCP2 and total MeCP2 in C6 cells exposed to NGF and BDNF for 1 h after 24 hours of serum starving. (B) Graph shows increased levels of pS421MeCP2 in BDNF and NGF treated groups. (C) Graph shows increased levels of pS80MeCP2 in BDNF and NGF treated groups. (D) Western blot analysis was applied to the cytoplasmic and nuclear proteins of the control and neurotrophins treated groups. MeCP2 and pS80MeCP2 were mainly found in the nuclear fraction, while pS421MeCP2 was found to be concentrated in cytoplasm in control group. GAPDH was used as control for the cytoplasmic protein, and H3 for the nuclear proteins. (E, F) After densitometric analysis, pS80MeCP2 and pS421MeCP2 data were normalized to the loading controls of cytoplasmic and nuclear fractions. (E) Graph shows pS80MeCP2 levels are high in nucleus in BDNF and NGF treated group as compared to control. (F) Graph shows pS421MeCP2 levels are high in nucleus in BDNF and NGF treated group as compared to control. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's t test or one-way ANOVA parameter where appropriate (*P < 0.05, **P < 0.01, ***P < 0.001)*

Fig 4.6

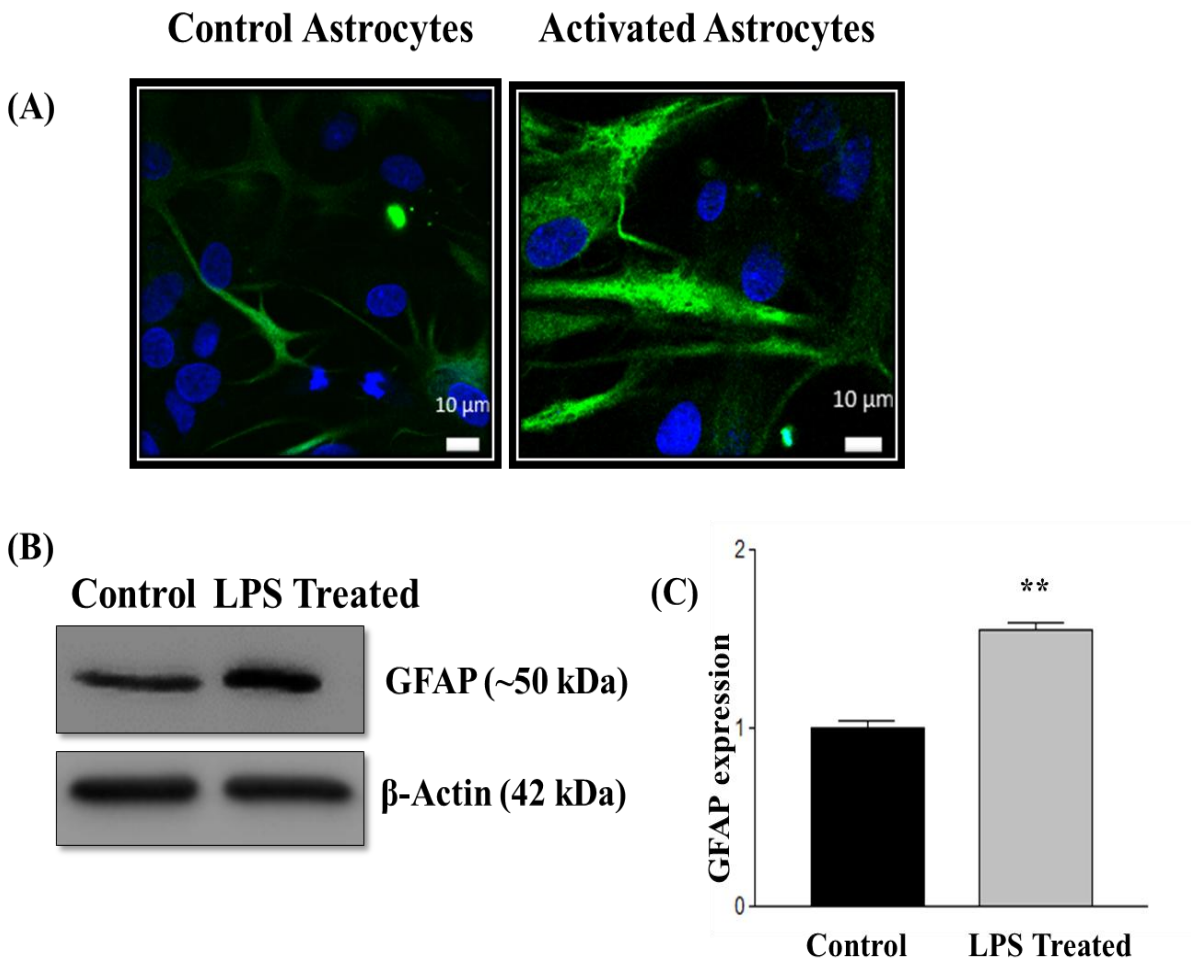


Fig 4.6 LPS treatment increases the GFAP expression in cortical astrocytes*(A)Representative immunostaining images showing GFAP expression (green) in Control and LPS treated Astrocytes. The nucleus was stained with DAPI (blue) Scale bar= 10µm. (B) Representative Western blot for GFAP in cortical astrocytes exposed to control and LPS. (B) Graph shows increased levels of GFAP in LPS treated group. Three different cell preparations were analyzed three times N= 3. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's t test or one-way ANOVA parameter where appropriate (*P < 0.05, **P < 0.01, ***P < 0.001)*

Fig 4.7

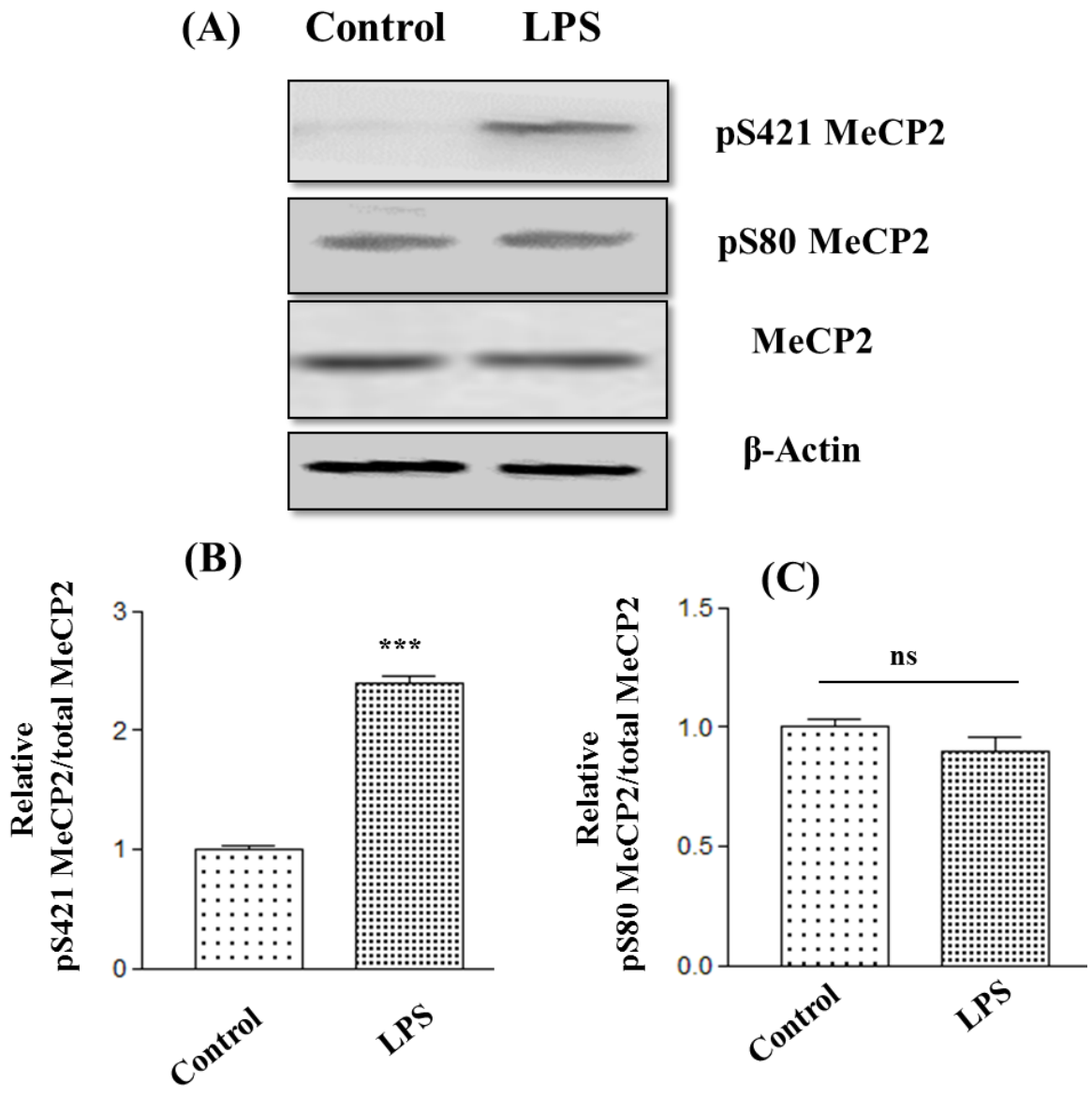

(A)
Control
LPS
pS421 MeCP2
pS80 MeCP2
MeCP2
β-Actin
(B)
Relative pS421 MeCP2/total MeCP2
***
Control
LPS
(C)
Relative pS80 MeCP2/total MeCP2
ns
Control
LPS

Fig 4.7

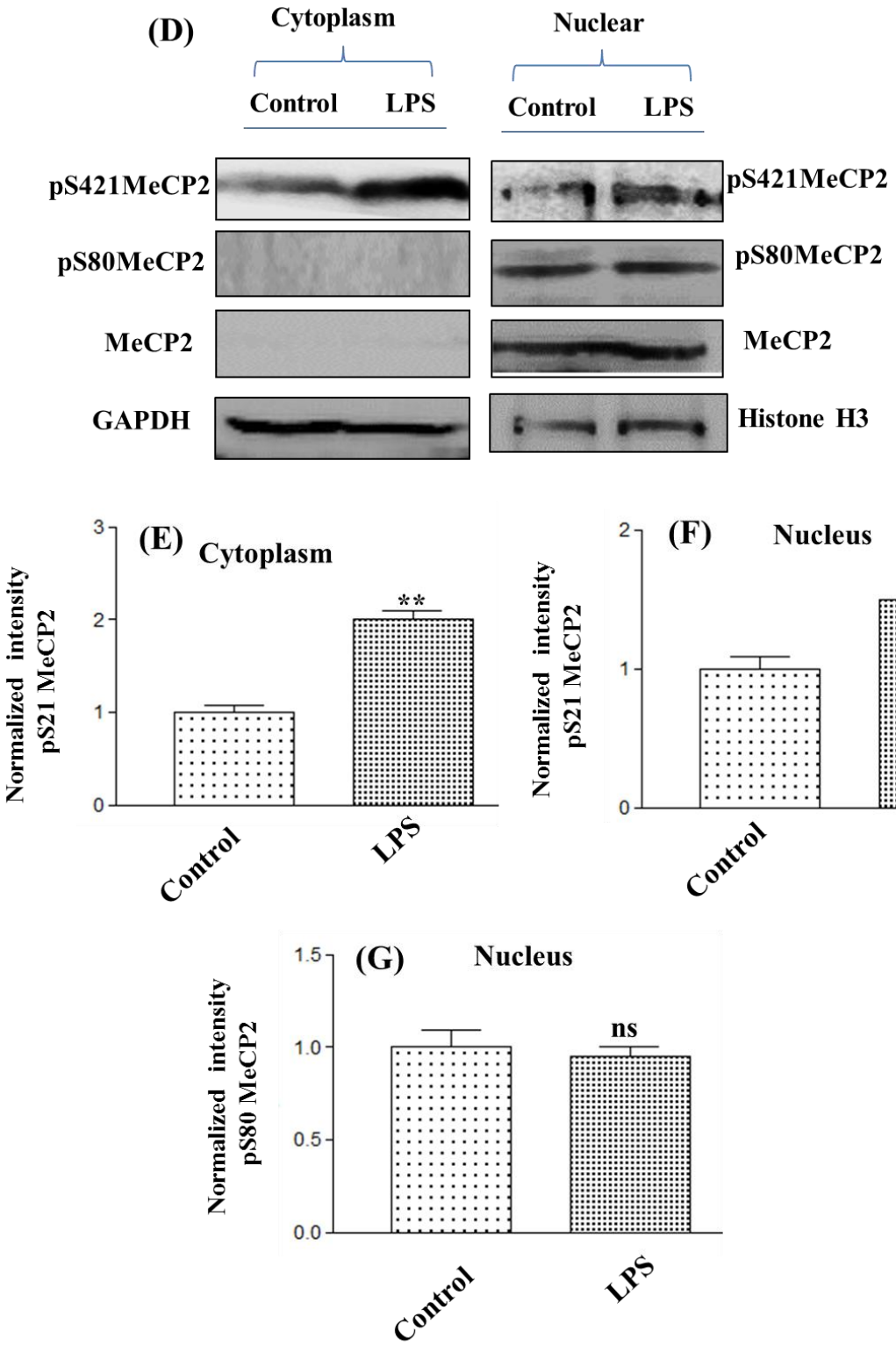

(D)
Cytoplasm
Nuclear
Control
LPS
Control
LPS
pS421MeCP2
pS80MeCP2
MeCP2
GAPDH
pS421MeCP2
pS80MeCP2
MeCP2
Histone H3
(E) Cytoplasm
Normalized intensity pS21 MeCP2
**
Control
LPS
(F) Nucleus
Normalized intensity pS21 MeCP2
*
Control
LPS
(G) Nucleus
Normalized intensity pS80 MeCP2
ns
Control
LPS

Fig 4.7 (H)

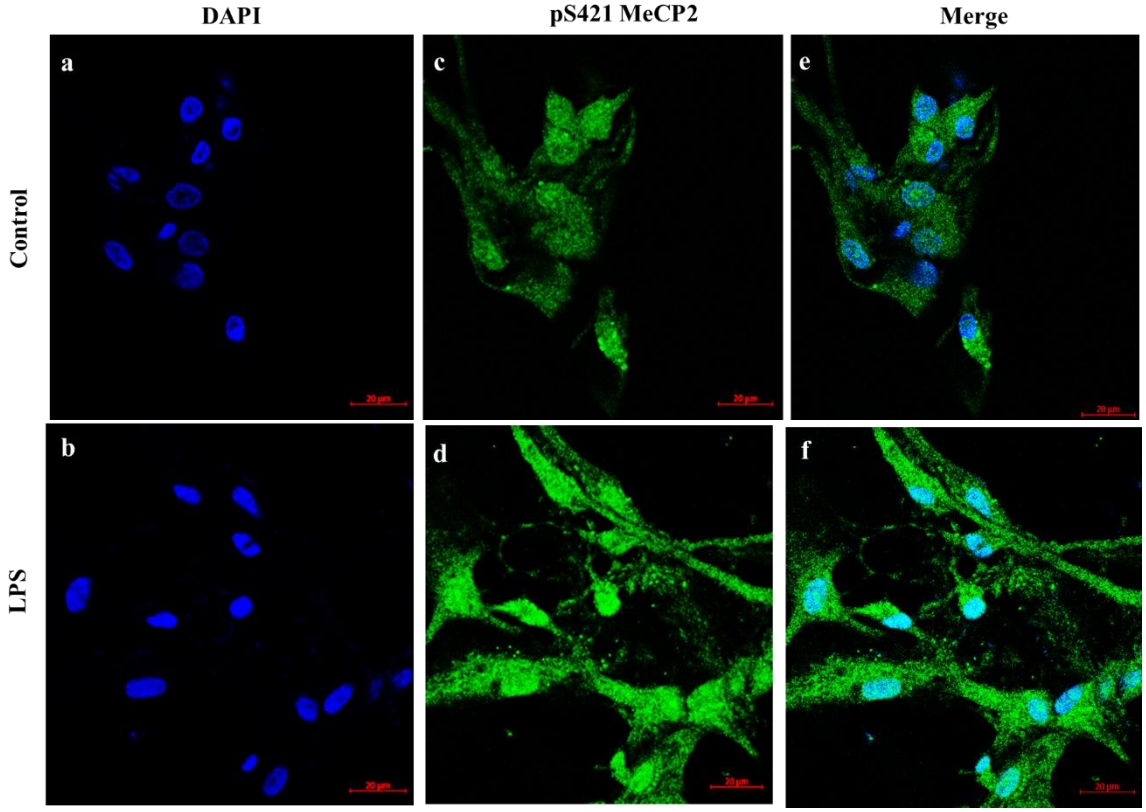


Fig. 4.7 MeCP2 phosphorylation by LPS in Astrocytes: *(A) Representative Western blot for pS80MeCP2, pS421MeCP2 and total MeCP2 in cortical astrocytes exposed to LPS for 4hrs after 24 hours of serum starving.(B) Graph shows increased levels of pS421 MeCP2 in LPS treated group.(C) Graph shows no change in the levels of pS80MeCP2 in LPS treated group (D)Western blot analysis was applied to the cytoplasmic and nuclear proteins of the control and LPS treated groups. MeCP2 and pS80MeCP2 were mainly found in the nuclear fraction, while pS421 MeCP2 was found to be concentrated in cytoplasm than nucleus. GAPDH was used as loading control for the cytoplasmic proteins, and H3 as a control for the nuclear proteins.(E-G) After densitometric analysis, pS421MeCP2 and pS80MeCP2 data were normalized to the loading controls of cytoplasmic and nuclear fractions. (E,F) Graph shows pS421 MeCP2 levels are high in nucleus in LPS treated group as compared to control. (G) Graph shows no significant difference in the pS80MeCP2 levels in the neurotrophins treated groups. (H)Representative immunostaining images showing pS421MeCP2 expression in astrocytes (green; c,d). The nucleus was stained with DAPI (blue; a,b). Colocalization of pS421 MeCP2 and DAPI shown in merge images (e,f). Three different cell preparations were analyzed three times N= 3. Values represent mean ± SEM. Statistical differences between the stimulated and control samples were determined using Student's t test or one-way ANOVA parameter where appropriate (*P < 0.05, **P < 0.01, ***P < 0.001)*