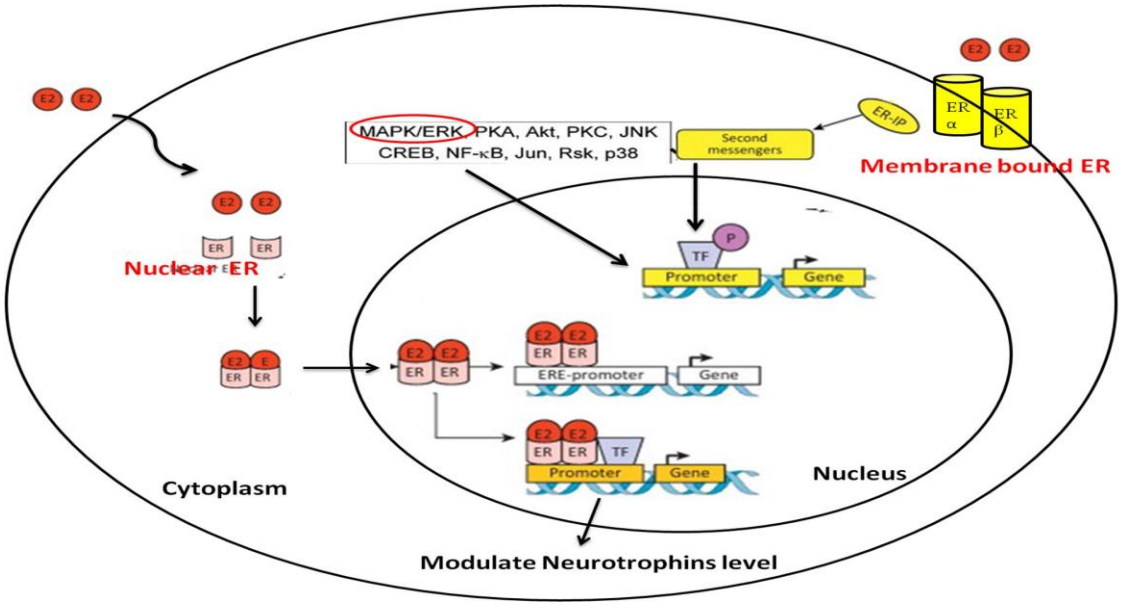
Chapter 6
To Study the mechanism underlying estradiol mediated neurotrophins expression in cortical astrocytes

6.1 Introduction:

Neurotrophins are essential growth factors for the regulation of CNS functions. There are four classes of Neurotrophins classified in human. Nerve growth factor (NGF), Brain derived neurotrophic factor (BDNF), Neurotrophins-3 (NT-3), Neurotrophins 4/5 (NT-4/5) (Allen & Dawbarn, 2006). These Neurotrophins exert their role by binding to their receptors, the Trk family of receptor tyrosine kinases (TrkA, TrkB, and TrkC) and p75NTR, a member of the tumor necrosis factor (TNF) receptor superfamily and triggers a complex series of signal transduction events, which are able to induce neuronal functions (Bucci, Alifano, & Cogli, 2014) and glial cells function. In CNS, both neuron and glial cells express neurotrophins and their receptors. Alterations in the expression of these neurotrophins leads to neuronal disorders as well as glial cells dysfunction such as Parkinson's disease (Howells et al., 2000; Sullivan & O'Keeffe, 2016; Sullivan & Toulouse, 2011) and Multiple sclerosis (Kalinowska-Lyszczarz & Losy, 2012; Razavi et al., 2015). Astrocytes are the most abundant glial cells and play a variety of complex and essential functions in the healthy CNS including house-keeping functions, providing nutrients and being markers of diseased conditions. Astrocytes are the main source for production of growth factors and neurotrophins (Sofroniew & Vinters, 2010). NGF and BDNF are most widely distributed in Astrocytes and are engaged in many of the essential functions of the brain such as neural protection (Allen, Watson, Shoemark, Barua, & Patel, 2013; Cheng & Mattson, 1994), neuron outgrowth (Hannan et al., 2015; Labelle & Leclerc, 2000), glial cell proliferation (Douglas-Escobar, Rossignol, Steindler, Zheng, & Weiss, 2012; Tsiperson et al., 2015) and myelination (Chan et al., 2004; Xiao et al., 2010). Astrocytes growth factor secretion function is affected by many instructive and extractive factors, hormones are one of them. 17-β estradiol, 17β-estradiol (E2), a steroid hormone plays a diverse role at cellular and molecular levels in brain cells. Secretion of this hormone is mainly by the ovaries, brain and fat tissue through aromatization of testosterones. During development, estradiol regulates cell proliferation, differentiation and survival in the brain (MacLusky, Clark, Naftolin, & Goldman-Rakic, 1987; Toran-Allerand, 2005). Estradiol regulates neural plasticity and dendritic spine density in the

brain region (Gould, Woolley, Frankfurt, & McEwen, 1990; Matsumoto & Arai, 1981; Woolley, Gould, Frankfurt, & McEwen, 1990). Two distinct estrogen receptors (ERs), ER alpha (ERα) and ER beta (ERβ) are distributed in the brain regions (McEwen, Alves, Bulloch, & Weiland, 1997; Mitra et al., 2003; Register, Shively, & Lewis, 1998; Shughrue, Lane, & Merchenthaler, 1997) and located in nucleus as well as in cytoplasm. Mode of action of these receptors is as per its localization (Lee & McEwen, 2001). Nucleus-initiated receptor signaling activates new gene transcription by association with estrogen response elements (EREs) in the DNA (Nelson & Bulun, 2001), also called direct genomic action, while membrane associated receptors cooperate with growth factor receptors or G protein-coupled receptors to activate kinase cascades through indirect genomic action (Hammes & Levin, 2007; Levin, 2005; Thomas, Pang, Filardo, & Dong, 2005; Vasudevan & Pfaff, 2007). However, role of E2 in the modulation of NGF and BDNF in cortical astrocytes and the mechanism driving this modulation is still not well understood. Thus, in the present study, we demonstrate the neurotrophomodulatory effect of E2 through its receptors. We have also studied the involvement of ERK signaling cascade in this modulation along with assessing the effect of E2 in astrocytes cell survival. Present study will help in understanding of neuroprotective role of E2 and its action in neurodegenerative disorders such as demyelination.



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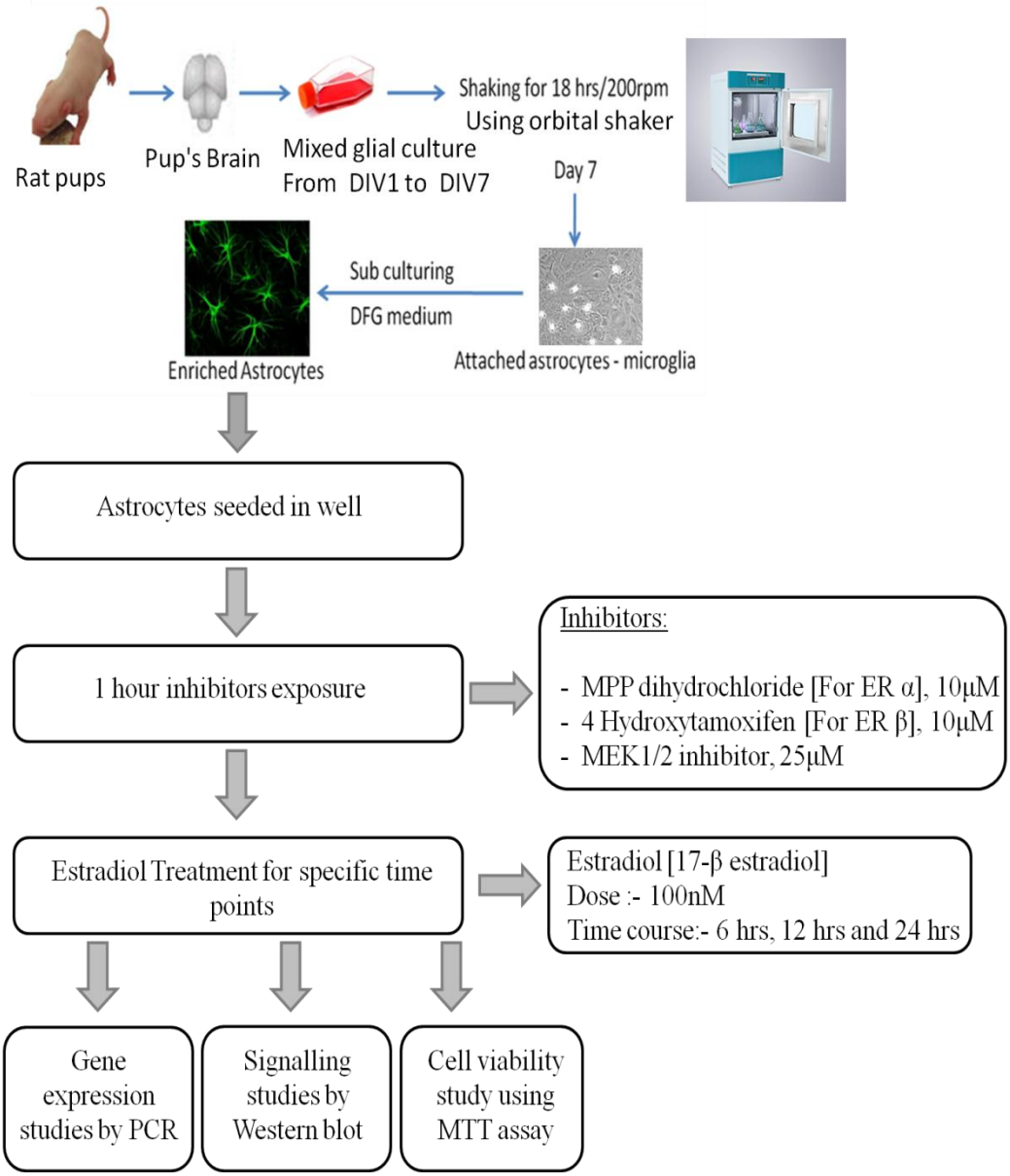
6.2. Strategy of work:



6.3 Results:

6.3.1: Isolation and characterization of rat cortical Astrocytes

Astrocytes were cultured by a previously described method (Chen et al. 2007; Sharma et al. 2015; Tripathi et al. 2017). Astrocytes culture was isolated from P0–2 day rat cortices. In current protocol, pups were sacrificed and cortical region of the brain was isolated for establishing astrocytes culture. The isolated astrocytes were significantly enriched and confirmed using morphological and Immunocytochemical analyses. Here results indicate phase contrast and confocal microscopic imaging of rat cortical astrocytes cells (Fig. 6.1).Adherent astrocytes were dissociated with 0.1%Trypsin and passaged using the same medium for further experiments.

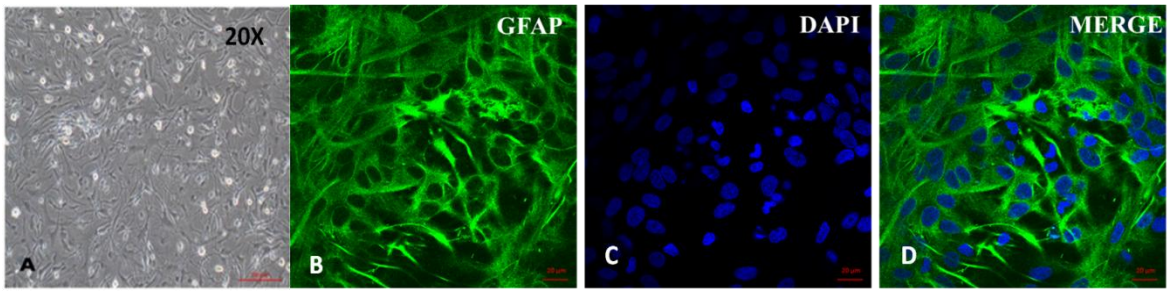


Figure 6.1: Morphological and Immunocytochemical characterization of Astrocytes: *Dissociation of 0–2 days old neonatal rat cortex yields predominantly GFAP positive astrocytes in primary culture following fibroblast removal.(A) Phase contras microscopy image of confluent astrocytes. (B) 63X immunofluorescent microscopy using Rabbit Anti-GFAP (Green) monoclonal antibody visualized with (D) Anti Rabbit FITC secondary antibody and (C) DAPI stain for nucleus identification. Scale Bar = 20 µm*

6.3.2: 17-β Estradiol modulates NGF and BDNF transcript levels in cortical Astrocytes

Astrocytes were cultured by a previously described method (Chen et al., 2007; Sharma, Singh, Pillai, & Frost, 2015; Tripathi, Parikh, Vora, Frost, & Pillai, 2017). Mixed glial cultures were harvested from P0-P2 day rat pups. After 10 days of culturing, astrocytes

were isolated from mixed glial culture. Isolated astrocytes were plated in DMEM containing 10% FBS and 1% penicillin/streptomycin on PLL coated 12 wells culture dish. After reaching confluency (1 x 10^6 cells) astrocytes were pre-incubated as follows : 10μM ERα antagonist (MPP Dihydrochloride; Sigma;) or 10μM ERβ antagonist (4-Hydroxytamoxifen; Sigma) (Bains, Cousins, & Roberts, 2007; Dong, Song, Xie, & Jiang, 2009) for 1 hour followed by treatment with 100nM 17β-Estradiol (1, 3, 5-Estratreine-3,17β-diol; HiMedia) (Kajta, Trotter, Lasoń, & Beyer, 2006; Lee & McEwen, 2001; Lorenzo, Diaz, Carrer, & Caceres, 1992) for three different time points- 6hrs, 12hrs and 24hrs. Results indicate up-regulation of neurotrophins mRNA expression levels for all time points which indicate that E2 positively regulates neurotrophins transcript levels. To further validate this, E2 receptors were blocked using specific inhibitors- MPP Dihydrochloride (ERα inhibitor, 10μM) and 4-Hydroxytamoxifen (ERβ inhibitor, 10μM). Cells were incubated with these inhibitors for 1 hour followed by E2 treatment for three different time points- 6hrs, 12 hrs and 24 hrs. In 6hrs time point, NGF was found to be up-regulated by E2 treatment, with a corresponding decrease as seen in E2 receptor inhibitor treated groups (both ERα and ERβ). On the other hand, BDNF was up-regulated after E2 treatment but surprisingly in the receptor inhibitor groups, the BDNF levels were not significantly declined (Fig. 6.2.A, 6.2.B).While in 12 and 24 hrs time point, both NGF and BDNF levels were found to be elevated in E2 treatment group with a corresponding decrease in E2 receptor inhibitor treated groups (ERα and ERβ) (Fig. 6.2.C, 6.2.D, 6.2.E, 6.2.F) which indicates a crucial role of E2 and its receptors in the regulation of neurotrophins in cortical astrocytes.

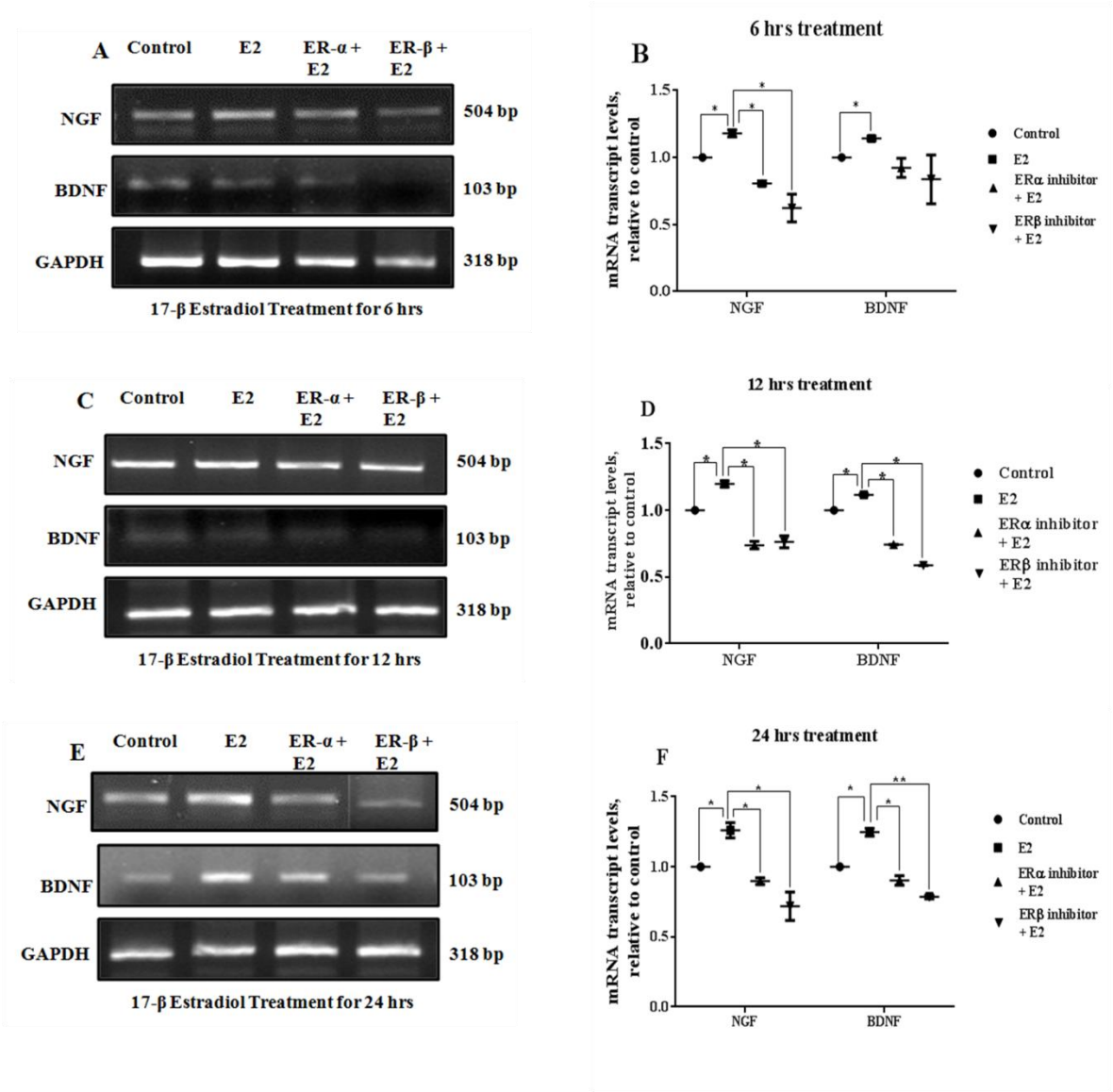


Figure 6.2: Effect of 17β- Estradiol on NGF and BDNF mRNA levels in rat cortical astrocytes cultures: *The cells were treated with 100nM Estradiol dose for 6hrs, 12hrs and 24 hrs, along with receptor inhibitors (10μM) and the NGF and BDNF mRNA levels were analysed by RT-PCR. Typical pattern of RT-PCR products for NGF, BDNF and GAPDH are indicated for 6 hrs (A), 12 hrs (C) and 24 hrs (E). Time course changes in NGF and BDNF mRNA transcript levels after 6 hrs (B), 12 hrs (D) and 24 hrs (F). mRNA expression levels of NGF and BDNF were normalized to GAPDH mRNA transcript*

*levels. E2 alone group was compared with control and ER-α or β inhibitor groups separately. Results are expressed as Mean ± SEM of three independent experiments and the data were evaluated using One way ANOVA followed by Bonferroni test (**p < 0.01, *p<0.05).*

6.3.3: Involvement of ERK in E2 mediated modulation of NGF and BDNF transcript levels:

To study the mechanism of E2 in modulation of NGF and BDNF, we studied the involvement of ERK. Isolated astrocytes were plated in DMEM containing 10% FBS and 1% penicillin/streptomycin on PLL coated 12 wells culture dish. Astrocytes were pre-incubated with 25μM MEK inhibitor (PD098059; Invitrogen) (Blázquez, Galve-roperh, & GuzmÁn, 2000; Choi, Ni, & Jonakait, 2011) for 1 hour followed by treatment with 100nM 17β-Estradiol (1, 3, 5-Estratreine-3,17β-diol; HiMedia) (Kajta et al., 2006; Lee & McEwen, 2001; Lorenzo et al., 1992) for three different time points- 6hrs, 12hrs and 24hrs. The time course for the analysis was decided to follow both the genomic and non-genomic action of estradiol (Lee & McEwen, 2001). Lee and McEwen (2001), reported that the mode of action of E2 is through indirect genomic action also,which occurs by activating secondary messenger cascade. Results showthat E2 induced neurotrophins levels were not found to be inhibited by MEK inhibitor (25μM) indicating that the possible mode of E2 action is the direct genomic action (Fig. 6.3.A, 6.3.B). However, in 12hrs and 24 hrs(long term exposure), pre-treatment with MEK inhibitor caused down-regulation of NGF and BDNF levels, thus suggesting that ERK might be involved in E2 mediated modulation of neurotrophins through the indirect genomic action (Fig. 6.3.C, 6.3.D, 6.3.E, 6.3.F).

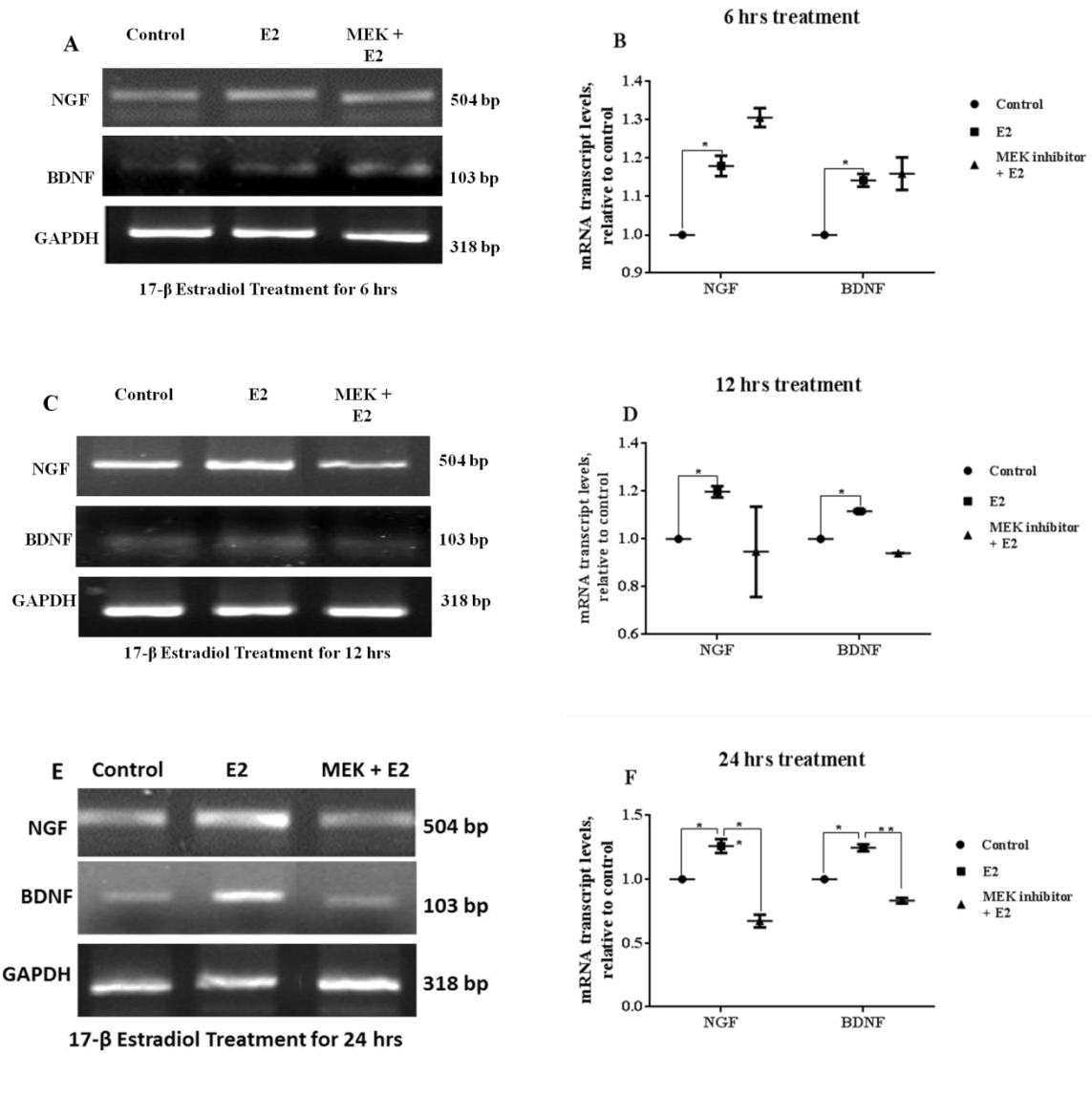


Figure 6.3: ERK involvement in E2 mediated modulation of NGF and BDNF transcript levels: *The cells were treated with 100nM Estradiol dose for 6hrs, 12hrs and 24 hrs, along with MEK inhibitor (25μM) and the NGF and BDNF mRNA levels were detected by RT-PCR. Typical pattern of RT-PCR products for NGF, BDNF and GAPDH are indicated for 6 hrs (A), 12 hrs (C) and 24 hrs (E). Time course changes in NGF and BDNF mRNA transcript levels after 6 hrs (B), 12 hrs (D) and 24 hrs (F).mRNA expression levels of NGF and BDNF were normalized to GAPDH mRNA transcript*

*levels. E2 alone group was compared with control and MEK inhibitor groups separately. Results are expressed as Mean ± SEM of three independent experiments and the data were evaluated using One way ANOVA followed by Bonferroni test (**p < 0.01, *p<0.05).*

6.3.4: Modulatory effects of E2 and its receptors on p-ERK1/2:

ERK is a well studied secondary messenger involved in directing cellular responses to a diverse array of stimuli. We aimed to understand the mode of action of E2 as a neurotrophomodulator in astrocytes.To justify this aim, astrocytes were treated with E2 receptors inhibitors (10μM) for 1 hour followed by treatment of E2 (100nM) for 6 hrs, 12 hrs and 24 hrs. Post treatment protein lysate was collected to perform western blot of p-ERK1/2. Results indicate in 6 hours treatment p-ERK1/2 levels are greatly affected by ERβinhibition compared to control andonly E2 treatment group (Fig. 6.4.A, 6.4.D). Surprisingly in 12 hours, inhibition of ERα increased p-ERK levels but not significantly while ERβinhibition causes drastic down-regulation of p-ERK levels (Fig. 6.4.B and 6.4.E). However in 24 hours treatment, E2 upregulated the phosphorylation of ERK1/2 compared to control while ERβ inhibition significantly downregulated the levels of p-ERK 1/2 compared to control and only E2 treatment groups (Fig. 6.4.C and 6.4.F). Thus, overall results demonstrate that p-ERK1/2 levels are down-regulated by ERβinhibition in all three time points, which indicates ERK acts as a secondary molecule for E2 mediated neurotrophin modulation via ERβ receptor.

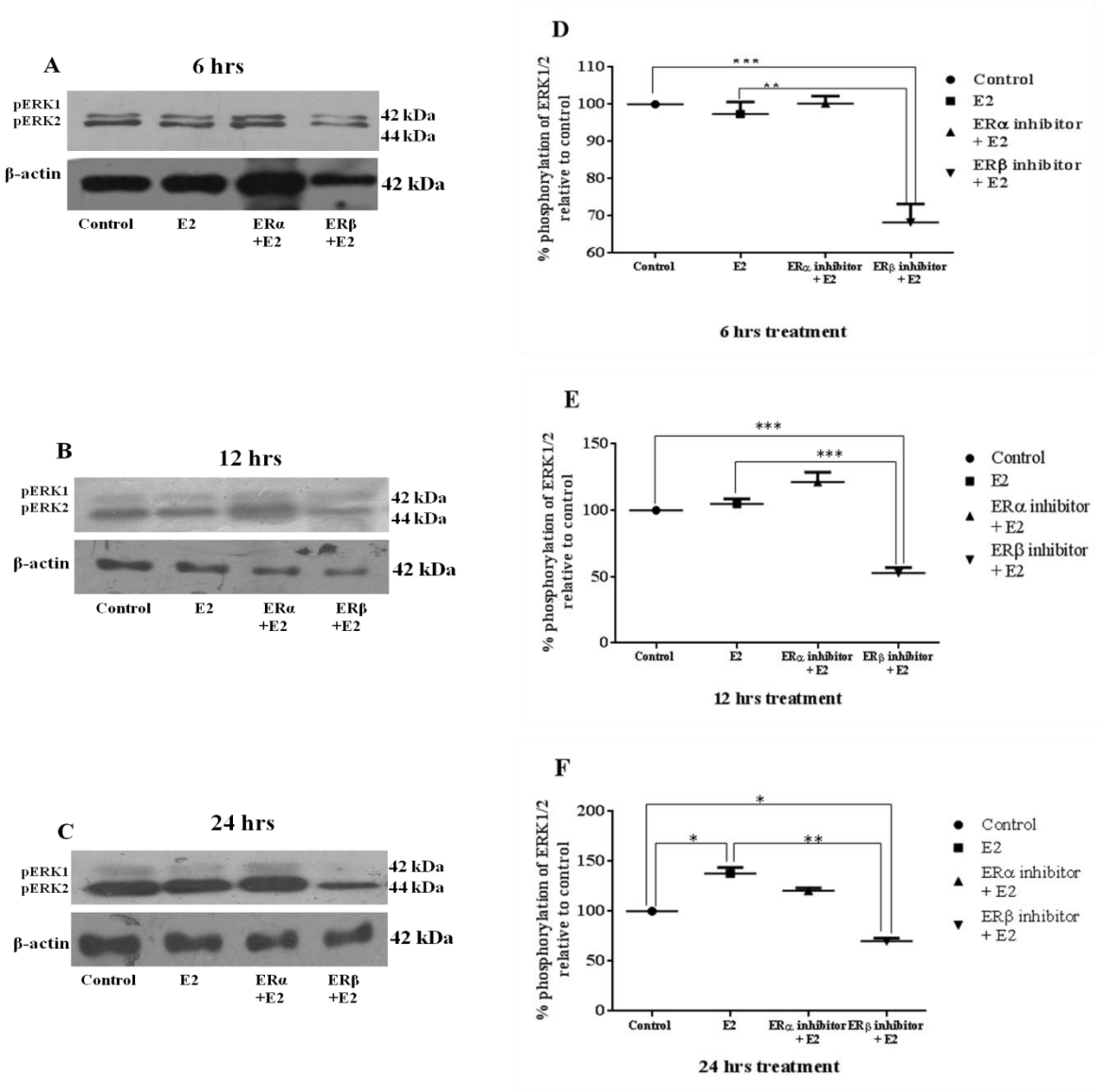


Figure 6.4: Modulatory effect of E2 and its receptors on p-ERK1/2*: Cultured astrocytes were harvested at 6, 12 and 24 hrs after E2 along with ERs inhibitors' treatment. Western blotting analysis of proteins extracted from astrocytes was performed using antibodies against p-ERK1/2 [6hrs (A and D), 12 hrs (B and E) and 24hrs (C and F)]. Phosphorylation of ERK1/2 were normalized to β-actin protein levels and mean values in control groups were scaled to 100%. Results are expressed as Mean ± SEM of three*

*independent experiments and the data were evaluated using One way ANOVA followed by Bonferroni test (***p<0.001, **p < 0.01, *p<0.05).*

6.3.5: ERα and ERβ mediated cell Viablity:

The growth stimulatory effects of 17β-estradiol and its receptors on cortical astrocytes cells was determined by a colorimetric assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Riss et al., 2016) that detects the formation of dark blue formazon from MTT in active mitochondria. In brief, cortical astrocytes were treated with 17-β estradiol with and without ER-α or ER-β inhibitor for 24 hours. MTT assay was performed as described in Chapter 2 (Material and Method). Results suggested that the percentage viablity of astrocytes increased upon incubation with 100nM 17β-estradiol compared with the untreated control. Astrocytes were treated with ER inhibitors for 1 hour followed by E2 treatment for 6hrs, 12hrs and 24hrs, after which MTT assay was performed. Results demonstrate that the percentage viablity was significantly reduced in inhibitor treated groups compared to E2 and control (Fig. 6.5).

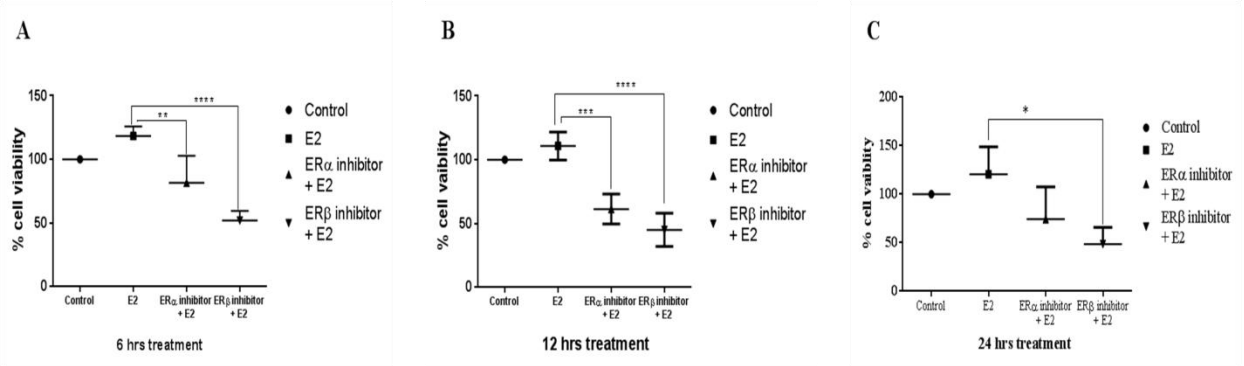


Figure 6.5: ERα and ERβ mediated cell viablity: *Cell viability after 6 hrs, 12 hrs and 24 hrs of E2 treatment along with receptors inhibitor respectively (A, B and C). Cell viability was measured by MTT assay. E2 alone group was compare with control and ER-α or β inhibitor groups separately. Statistical analysis was performed using one way ANOVA followed by Bonferroni test (***p<0.001, **p < 0.01, *p<0.05).*

6.3.6: Role of ERK in E2 treated astrocytes cell viablity:

The growth stimulatory effects of 17β-estradiol and its receptors on cortical astrocytes was determined by a colorimetric assay using MTT. In brief, cortical astrocytes were treated with 17-β estradiol with or without MEK inhibitor for 24 hours. MTT assay was performed as described in Chapter 2 (Material and Method). Results demonstrate that there was no significant difference in the E2 treated groups and MEK inhibitor treated groups indicating that ERK does not play any role in cell viablity (Fig. 6.6).

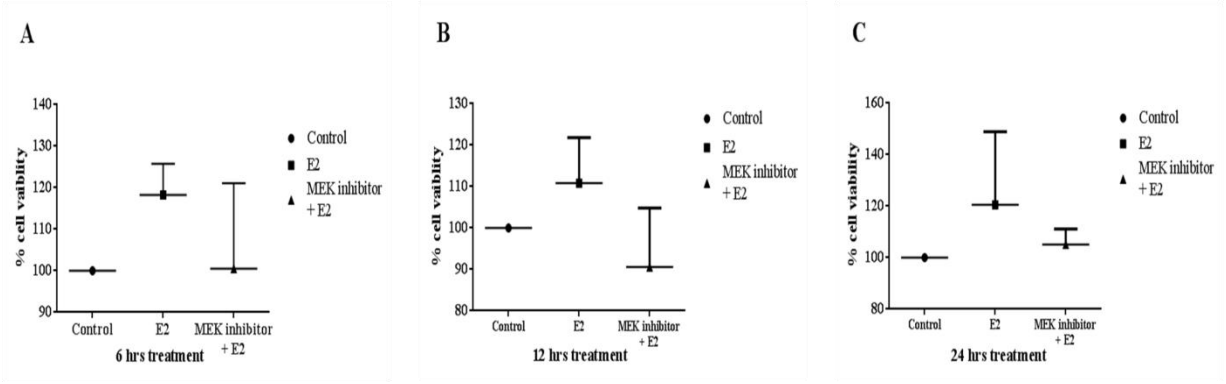


Figure 6.6: Role of ERK in E2 treated astrocytes cell viablity: *Cell viability after 6 hrs, 12 hrs and 24 hrs E2 treatment along with MEK inhibitor (A, B and C). Cell viability was measured by MTT assay. E2 alone group was compare with control and MEK groups separately. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test.*

6.4: Discussion:

In all complex nervous systems neuronal cells coexist with glial cells. Astrocytes are major and most abundant glial cells in the CNS which play a major role in a number of house-keeping and critical functions of neurons and oligodendrocytes such as neuronal maturation, neuronal survival, oligodendrocyte maturation and support to the myelination milieu. This suggests that neuron-glia interactions are principal features for proper functioning of both types of cells. The principal neuro-glia interaction is myelination.

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Astrocytes are found to be closely associated with axons at an early stage of their myelination by oligodendrocytes (Ioannidou, Anderson, Strachan, Edgar, & Barnett, 2014) and induce oligodendrocytes to align their processes with axons (Meyer-Franke, Shen, & Barres, 1999). Astrocytes are known to secrete many growth factors, neurotrophins, ECM molecules which are reported to influence myelination which were reviewed and compiled (Barnett & Linington, 2013). Certain neurotrophins (Miyamoto et al., 2015) secreted by astrocytes are crucial for timing oligodendrogenesis, development and function. They induce oligodendrocytes to align their processes with axons (Meyer-Franke et al., 1999). BDNF is a well-known pro-myelinating factor secreted by astrocytes. These Functions of the Astrocytes are affected by many instructive and extractive factors, hormones are one of them. 17β-estradiol (E2), a steroid hormone plays a diverse role at cellular and molecular levels in brain cells. Neurons in the adult rat forebrain of both sexes co-express estrogen and neurotrophin receptors and are also the sites of estrogen and neurotrophin synthesis (Miranda, Sohrabji, & Toran-Allerand, 1994; Toran-Allerand et al., 1992). It has also been shown that estradiol and neurotrophin receptor co-expression leads to convergence of their signaling pathways (Toran-Allerand, Singh, & Sétáló, 1999). Thus, it was interesting to explore the involvement of estradiol in neurotrophin expression in the astrocytes, whether the estradiol mediated modulatory effects on neurotrophins are mediated via direct or indirect genomic actions and to study the signalling pathway, particularly the ERK signalling pathways, activated by estradiol receptors.

17β estradiol, (through both ERα and ERβ), contributes to the modulatory levels of neurotrophins (NGF & BDNF) and our data suggests ERβ is majorly involved and responsible for cortical astrocytes cell growth, proliferation and thus play an important role in neuroprotection. Estradiol induced neurotrophin level in astrocytes was reduced after blocking the ERs which confirms the role of estradiol in the modulation of neurotrophin expression. Estradiol induced neurotrophin expression levels following exposures to E2 (6, 12 and 24 hours) and in the case of 24 hrs exposure to E2, neurotrophin expression levels post MEK inhibitor was significantly reduced which

indicates ERK might be involved in estradiol signalling pathway through indirect genomic action. Protein analysis shows ERK1/2 act as a secondary molecule for estradiol mediated neurotrophin modulation, via indirect genomic action and is activated in long term E2 exposure condition for astrocytes survival. Present data strongly indicates ERβ may be majorly responsible in modulating the level of neurotrophins and thus responsible for astrocytes cell survival compared to ERα.

ER and neurotrophin receptors co-localizations are widespread in neurons of the cerebral cortex, hypothalamus and hippocampus (Sohrabji & Lewis, 2006) which explain the fact that estradiol and neurotrophins generally appear to exert reciprocal regulation upon each other's actions at the level of gene transcription. Reduced levels of E2 in post-menopausal women coincide with the high rate of neurodegenerative disorders in this age group, as reported by clinical studies (Rocca, Grossardt, & Shuster, 2011). E2 therapy thus may be highly beneficial in protection against certain neurodegenerative diseases as Alzheimer's (Birge, 1997), Parkinson's (Adams & Kumar, 2013) and brain ischemia (Ma et al., 2016). Moreover, NGF and BDNF are well studied with respect to their role in treatment of neurological disorders (Balaratnasingam & Janca, 2012; Cattaneo & Calissano, 2012). Thus, estrogen and neurotrophin receptors crosstalk gives rise to a possible therapeutic approach of neuroprotection against neurodegenerative diseases. We sought to understand the effect of E2 on NGF and BDNF in astrocytes in a time dependent fashion in 6, 12 and 24 hrs to elucidate the underlying cellular mechanism. Neurotrophins were significantly up-regulated in all the three-time points of E2 exposure in astrocytes compared to control. Next, we analyzed if this up-regulation was mediated by E2 receptors for which both ERα and ERβ were blocked using specific inhibitors (as described in the materials & methods section) wherein neurotrophin levels were significantly down-regulated in 12 and 24 hrs but not in 6 hrs. Our results indicate that E2 positively regulates the modulation of neurotrophins in astrocytes through both its receptors- ERα and ERβ.

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Estradiol receptors exist in two forms nuclear and membrane-associated and both of which are involved in mediating neurotrophic and neuroprotective estrogen effects. Nuclear or direct genomic actions are independent of secondary messenger whereas non-nuclear or indirect genomic estrogen effect activation of second messenger cascades and transcription factors that are regulated via phosphorylation and de-phosphorylation, some of which are also linked to cells survival. Moreover, estrogen and neurotrophins stimulate a common secondary messenger- ERK (Lee & McEwen, 2001). In cortical explants cultures, estrogen phosphorylates the MAP kinases ERK1 and ERK2 in a time frame similar to that of the neurotrophins (Singh, Sétáló, Guan, Warren, & Toran-Allerand, 1999).In the present study, we tried to understand the mode of E2 action in modulation of neurotrophins levels in astrocytes. To justify this aim, phosphorylation of ERK1/2 was checked to confirm the secondary messenger activation. We found significantly down regulation of p-ERK1/2 levels in ERβ inhibition group in all time points which indicating action of E2 was mediate by ERK pathway only through indirect genomic action. Overall, these results indicate that ERβ mediated ERK is involved in E2 modulated neurotrophins expression through the indirect genomic action.

In conclusion, 17β-estradiol (E2) acts as a neurotrophomodulator and regulates viability of cortical astrocytes. Estradiol receptor β (ERβ) play a vital role in mediating neurotrophomodulator functions of E2. We also show that E2 mediated modulation of neurotrophins is via ERβ mediated ERK signalling pathway indicating the indirect genomic action of this hormone. Thus, crosstalk between neurotrophins and E2 play a very significant role on glial cells' function and survival suggesting its potential benefits as a neuroprotector in neurodegeneration disorders such as multiple sclerosis.