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17BETA-ESTRADIOL, INTEGRINS AND SYNAPTIC PROTEINS

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Manjari Chandra, B.S.

Fort Worth, Texas

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CHAPTER I

INTRODUCTION

A. Oxidative stress and neurodegenerative diseases

Neurodegenerative diseases involve deterioration of neurons which over a period of time affects many brain-related functions such as cognition and memory. Oxidative stress is implicated in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke. The free radical theory of aging presents reactive oxygen species (ROS) as an important player in many diseases (Harman, 1992). The brain is particularly vulnerable to oxidative damage due to its high utilization of total body oxygen of (20%) even though it represents only 2% of the body weight. This energy requirement is necessary to maintain the ion gradients across the plasma membrane which is important for the generation of action potentials. Also, the brain is composed of large amount of easily oxidizable polyunsaturated fatty acids and is relatively deficient in antioxidant defense systems. Majority of ROS are generated during the process of oxidative phosphorylation that takes place in the mitochondrial membrane. Some electrons that escape from the electron transport chain react with oxygen molecule to form free radicals such as singlet oxygen and superoxide anion ($O_2^{\cdot-}$). The free radicals generated continue to form more free radicals by chain reactions involving various red-ox enzymes. The excess ROS generated can overwhelm the antioxidant defense systems and potentially damage cellular components including proteins, lipids and DNA leading to oxidative damage (Lin and Beal, 2006). Figure 1 show a schematic representation of various enzymatic

and non-enzymatic antioxidant defenses and the ROS involved in causing the oxidative damage (Sayre et al., 2008).

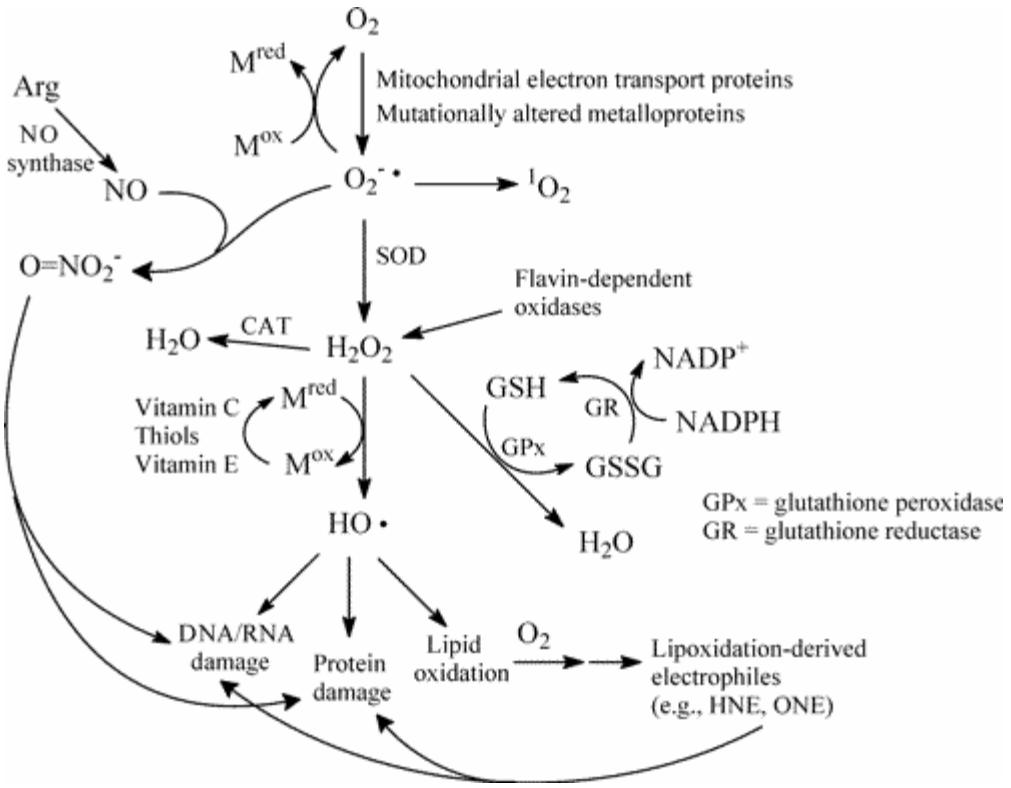

Fig 1: Schematic representation of various red-ox reactions taking place in the mitochondria (Sayre et al., 2008).

Hydrogen peroxide (H_2O_2) is one of the major ROS generated during the process of mitochondrial respiration from dismutation of superoxide ($O_2^{\cdot-}$) by the enzyme superoxide dismutase. The generated H_2O_2 is transformed to hydroxyl radical (·OH) through Fenton reaction with reduced transition metal ions (usually Fe^{2+} and Cu^{+}). The hydroxyl radical (·OH) is highly reactive and is considered the main instigator of oxidative damage in the cellular system by reacting with all biomacromolecules (Sayre et al., 2008). There is abundant evidence that amyloid-β peptide induces oxidative stress and neurotoxicity in AD (Butterfield, 2002, Varadarajan et al., 2000). Though the mechanisms behind Aβ-induced oxidative stress are less clear, studies have shown H_2O_2 as the chief mediator of amyloid-β peptide induced neurotoxicity

(Behl et al., 1994). The full length Aβ peptide ($A\beta_{1-42}$) consists of a Cu^{2+} binding domain and can reduce the bound Cu^{2+} to Cu^{+}. The $A\beta_{1-42}$-associated Cu^{+} was reported to lead to the generation of H_2O_2 and thereby causing oxidative stress (Huang et al., 1999a, Huang et al., 1999b). H_2O_2 is also generated from dopamine oxidation by monoamine oxidase A/B in the brain (Palumbo et al., 1999). Overall, the oxidative stress mechanisms leading to cell death and the various targets of oxidative stress among the many bio-molecules that are essential or detrimental for the cell survival still need to be identified.

B. Estrogens and its neuroprotective actions

Previous studies from our laboratory have shown the protective effects of 17β-estradiol against H_2O_2-induced toxic effects such as lipid peroxidation, calcium overloading, ATP depletion, mitochondrial membrane potential collapse and ultimately cell death in cultured human lens epithelial cells and mouse neuroblastoma (SK-N-SH) cells (Wang et al., 2003, Wang et al., 2006). Estrogens are gonadal steroid hormones which have been shown to have neuroprotective properties against cellular dysfunction and damage. Many preclinical studies support the neuroprotective actions of estrogens against a variety of in vitro insults and in vivo challenges. The naturally occurring feminizing estrogen, 17β-estradiol has been shown to exert its neuroprotective effects against many in vitro toxic insults such as serum deprivation (Bishop and Simpkins, 1994), glutamate excitotoxicity (Singer et al., 1996), H_2O_2-induced toxicity (Behl et al., 1995, Green and Simpkins, 2000, Wang et al., 2006), amyloid-β peptide-induced toxicity (Green, Gridley and Simpkins, 1996, Gridley, Green and Simpkins, 1997) and various mitochondrial toxins (Wang, Green and Simpkins, 2001). Estrogens have also been shown to be potent neuroprotectants against a variety of models of acute cerebral ischemia (Simpkins et al.,

1997, Yang et al., 2001). There is evidence from clinical and epidemiological studies that estrogen therapy may contribute to prevention, attenuation or delay of the onset of Alzheimer's disease by improving cognition and memory deficits (Kawas et al., 1997, Paganini-Hill and Henderson, 1996). Early estrogen therapy is probably more beneficial in preventing or delaying the onset of neurodegenerative diseases based on studies such as WHIMS (Women's Health Initiative memory study) analysis (Shumaker et al., 2003, Shumaker et al., 2004).

Various mechanisms involved in estrogen-mediated neuroprotection have been described suggesting a complex, multifaceted process. The mechanisms by which estrogens exert their actions include genomic, non-genomic and mitochondrial mechanisms. It is known that estrogens exert most of their genomic actions through members of the nuclear hormone receptor super family estrogen receptor-alpha (ER-α) and estrogen receptor-β (ER-β). Upon estrogen binding to its receptor, ER-α and/or ER-β dimerizes and translocates to the nucleus. The activated receptor binds to specific DNA sequences in the promoter region of target genes to regulate transcription of those genes (Landers and Spelsberg, 1992). In addition, studies suggest the existence of membrane-associated estrogen receptors which have not been cloned yet. However, the rapidity of estrogen's actions in the brain cannot be completely explained by genomic mechanisms alone. Estrogens were demonstrated by many studies to activate a number of signal transduction pathways involved in regulation of cell survival. Some of these pathways include Ras/Raf/ERK (MAPK), the PI-3K/Akt, the cAMP/ PKA/CREB and the NFkB pathways (Driggers and Segars, 2002, Simpkins et al., 2005a, Wen et al., 2004). Several studies have indicated the important role played by mitochondria in estrogen-mediated neuroprotection. Estrogens either directly protect the mitochondria by interacting with estrogen-binding sites on the mitochondria or indirectly protect through regulation of signal transduction pathways thus

causing the inactivation of pro-apoptotic proteins (Nilsen and Diaz Brinton, 2003). Experimental studies have shown that estradiol treatment prevents the toxin-induced collapse of the mitochondrial membrane potential ($\Delta\psi m$) (Wang, Green and Simpkins, 2001). The maintenance of mitochondrial membrane potential prevents the mitochondrial swelling, release of cytochrome c and apoptotic factors thus preventing the apoptotic cell death (Simpkins et al., 2005b). The mitochondrial mechanisms of estrogen neuroprotection were reviewed in detail by (Simpkins and Dykens, 2008). Estrogen has also been shown to have direct antioxidant properties due to the presence of the phenolic-A ring in its structure. Estrogens can sacrifice the OH group to oxidation thus forming a quinol product, which is red-ox cycled back to the parent estrogen by utilizing the cellular reducing potential such as NADPH and glutathione, thereby acting as a defense mechanism against ROS (Prokai and Simpkins, 2007, Prokai et al., 2003).

C. Integrins

Integrins are a family of heterodimeric cell surface receptors that mediate both cell-cell and cell-matrix interactions in a wide variety of cell types (Aplin et al., 1998). The integrins are comprised of two subunits, α and β. Each subunit has a large extracellular portion, a single transmembrane segment and a short cytoplasmic domain (with the exception of β4). At least nine β subunits and eighteen α subunits have been identified and different α-β pairings give rise to 24 receptors with different ligand binding specificities. All the integrins fall into three basic classes: β1, β2 and αv (Milner and Campbell, 2002). Each integrin recognizes specific ligands which are either extracellular matrix (ECM) molecules such as laminin, collagen, fibronectin and vitronectin or cell surface counter-receptors of the immunoglobulin super family, such as intercellular adhesion molecule-1 (ICAM-1).

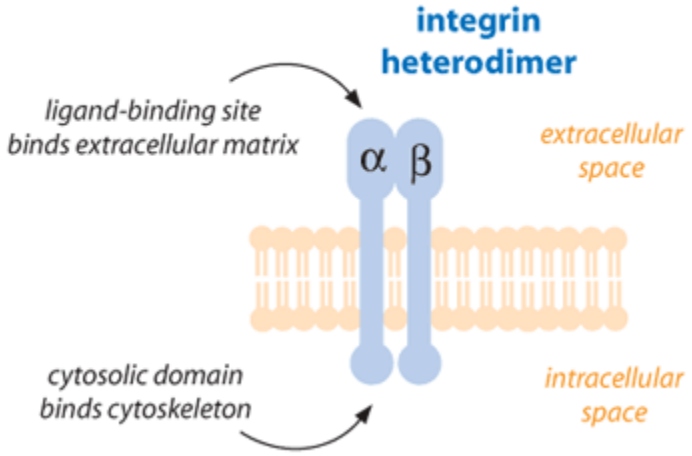

Fig 2: Structure of integrin heterodimer (http://www.scq.ubc.ca/wp-content/uploads/2006/08/integrins.gif)

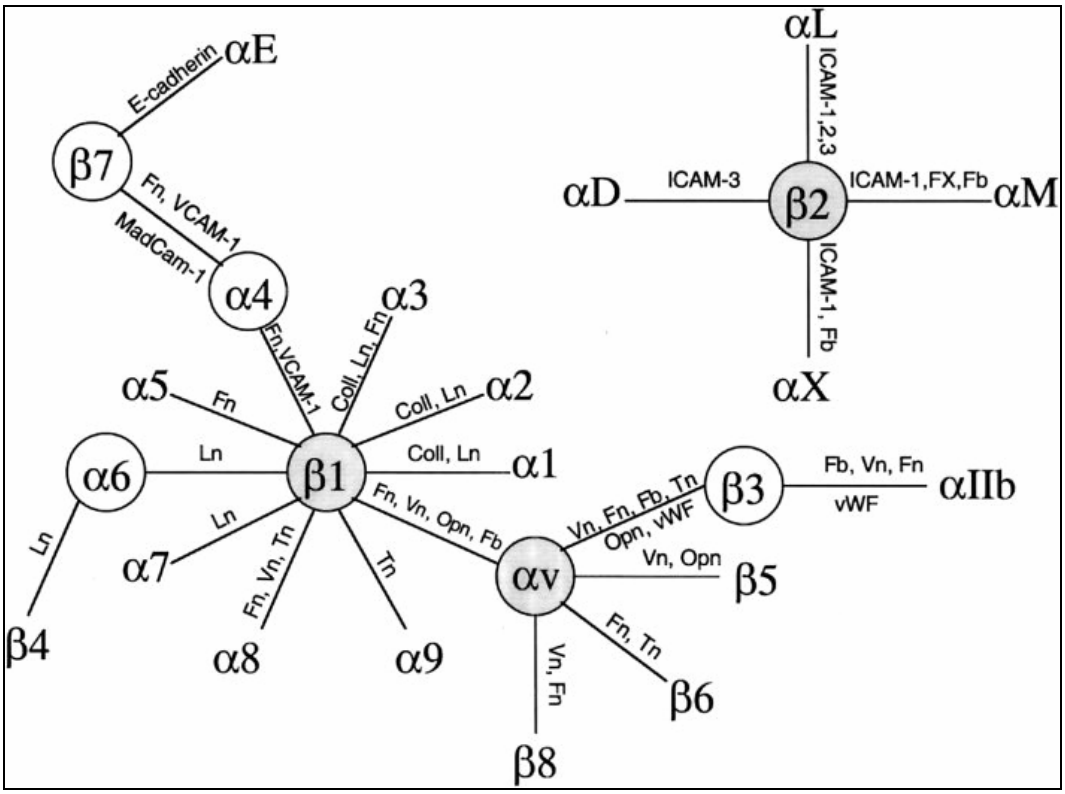

Fig 3: Integrin α-β subunits and their ligands. The shaded ones are the three major classes of integrins β1, β2 and αv (Milner and Campbell, 2002).

Integrins in the nervous system

Many types of integrin subunits are widely expressed in the nervous system. Integrins of β1 and αv classes are expressed on a variety of different cell types in central nervous system (CNS), including neurons, glia, meningeal cells and endothelial cells (Jones, 1996, Pinkstaff et al., 1999). β2 integrins are specifically expressed on microglia in the CNS and on infiltrating leukocytes (Jones, 1996). Two integrin subunits, α7 and α8 have been found to be specifically localized to the synapses (Einheber et al., 1996). Messenger RNAs encoding β1, 4 and 5 and α1, 3, 4, 5, 6, 7, 8, and v were reported to localize to different brain regions in an *in situ* analyses (Pinkstaff et al., 1999). There is evidence from many studies that integrins and neural cell adhesion molecules (NCAM) are involved in the events leading to long-term potentiation (LTP)

such as modulating synaptic architecture. The synaptic zone functions as the adhesive region between neurons and integrins. NCAMs play an important role in most of these adhesive interactions. Integrin-like proteins have been localized to synaptosomal membranes (Bahr and Lynch, 1992), and immunocytochemical studies with integrin antibodies have demonstrated synaptic-type staining in the hippocampus (Grooms et al., 1993; Jones, 1996). The infusion of RGD containing peptides into hippocampal slices disrupted the stabilization of long-term potentiation but induction of LTP was not blocked (Staubli et al., 1990; Bahr et al., 1997). Stabilization of LTP depends on the establishment of new adhesive contacts between cells, and cells and their matrix. Therefore, LTP must be related to a structural reconfiguration of the synaptic zone and integrins must be playing an important role in these events.

Integrin functions

Integrins in general are involved in a variety of cellular processes, such as, cell adhesion, migration, growth, survival, proliferation and differentiation. In the nervous system, integrins play an important role in the neuroblast migration, axon and dendrite outgrowth, synapse formation, learning and memory (Clegg, 2000, Milner and Campbell, 2002). Integrins are also involved in the CNS angiogenesis and stabilization of the blood brain barrier.

Role of integrins in signal transduction

Integrins can signal in either direction through the cell membrane. Binding of integrins to the ECM molecules elicits signals that are transmitted into the cell (outside-in signaling), while extracellular binding activity of integrins is regulated from the inside of the cell (inside-out signaling) (Aplin et al., 1998). Integrins by binding to ECM molecules activate various protein

tyrosine kinases including focal adhesion kinase (FAK), Src-family kinases and integrin–linked kinase (ILK). In addition to FAK, some β1 and αv integrins also activate Fyn and the adapter protein Shc. Through these interactions integrins activate the mitogen-activated protein kinase (Ras/ERK/MAPK) cascade and c-Jun NH2- terminal kinase (JNK), thereby regulating cell growth, proliferation and survival (Giancotti and Ruoslahti, 1999). Shc is responsible for the initial high level activation of ERK upon cell adhesion. The activation of ERK is sustained by FAK which is activated more slowly (Pozzi et al., 1998, Wary et al., 1998). Integrins may also contribute to protection from apoptosis because FAK binds PI-3K which in turn activates PKB/Akt that inactivates the pro-apoptotic proteins Bad and caspase-9. Signaling pathways activated by integrins and growth factor receptors are interconnected, most importantly at the level of Ras, PI-3K and FAK (Fig 3) (Giancotti and Ruoslahti, 1999). The coordinated control of cell shape, growth and survival by integrin signaling is important in the establishment and maintenance of tissue architecture.

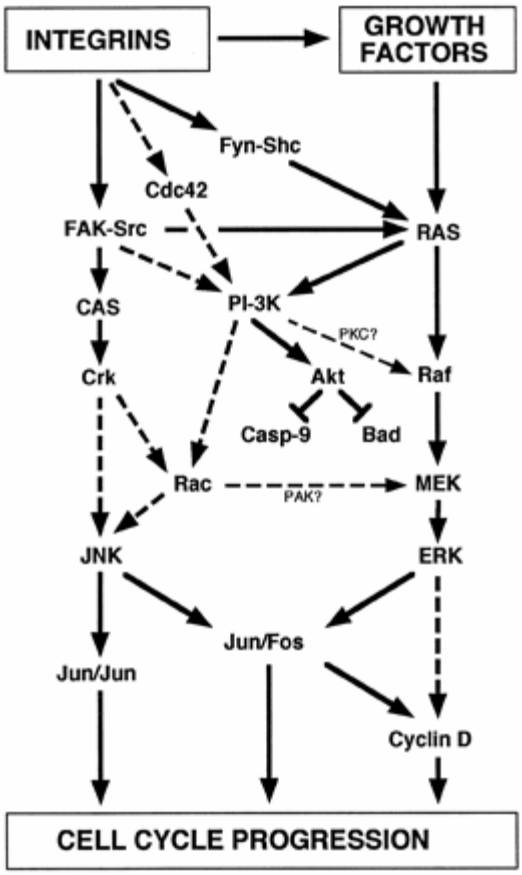

Fig 4: Major signaling pathways that are known (solid arrows) or presumed (dashed arrows) to be coordinately regulated by integrins and growth factor receptors (Giancotti and Ruoslahti, 1999).

More in depth investigations into integrins and integrin-mediated signaling reveals the role of integrin-mediated signaling in neuronal cell death pathways. Primary rat cortical neurons and human neuroblastoma (SH-SY5Y) cells exposed to amyloid-β peptide (Aβ $_{25\text{-}35}$) enters the cell cycle accompanied by modifications in the distribution of cells in different phases of the cell cycle. In addition, various cell cycle proteins are expressed subsequently, leading to neuronal apoptosis. The extracellular signal-regulated kinase (ERK) pathway was found to be involved in this effect. Interaction of Aβ peptides with integrins and the activation of FAK/Src were the events reported to take place upstream of ERK1/2 activation and subsequent cell death (Frasca et al., 2004). Therefore, the study demonstrated that integrins and the integrin-mediated signal

transducting pathways are involved in the Aβ induced neuronal death. Many studies have also reported the direct involvement of integrin subunits in Aβ induced-neurotoxicity. In human cortical neurons, integrins α2 β1 and αv β1 promote Aβ deposition and contribute to Aβ-neurotoxicity (Wright et al., 2007), and β1-integrin subunit is involved in Aβ stimulated generation of reactive oxygen species (Bamberger et al., 2003). Activation of FAK in dystrophic neurites surrounding senile plaques and neuronal cultures exposed to Aβ was reported suggesting a role for FAK in Aβ toxicity (Grace and Busciglio, 2003).

Evidence shows that upon oxidative stress or exposure to Aβ fibrils, fully differentiated and mature neurons in the adult brain exhibit cell cycle activation (Herrup et al., 2004, Kuan et al., 2004). It has been proposed that activation of integrin/FAK/FA (focal adhesion) pathway by oxidative stress or fibrillar Aβ induces neuronal death and concurrent activation of cell cycle proteins (Caltagarone, Jing and Bowser, 2007). Given the complexity of the role of integrins and integrin-mediated signaling in promoting either cell survival or cell death and the importance of integrins in synaptic plasticity, the role of integrins in normal physiological conditions as well as in diseased states must be clearly elucidated.

D. Significance

It is widely postulated that oxidative stress is one of the major factors involved in the etiology of various neurodegenerative diseases. Estrogens seem to be promising candidates in providing neuroprotection against a variety of insults *in vitro* and *in vivo*. Retrospective epidemiological studies in post-menopausal women indicate that hormone therapy (HT)/estrogen therapy (ET) is associated with a reduction in the risk of Alzheimer's disease as well as a delay in its progression (Paganini-Hill and Henderson, 1994, Paganini-Hill, Ross and Henderson, 1988,

Simpkins, Singh and Bishop, 1994, Tang et al., 1996). In addition, several clinical studies have reported improvement of cognitive functions in female AD patients receiving ET (Henderson et al., 1994, Ohkura et al., 1994). Moreover, epidemiological and clinical reports have shown diminished severity of Parkinson related symptoms in estrogen treated post-menopausal women with early PD (Sandyk, 1989, Saunders-Pullman et al., 1999). Clinical evidence has also shown that estrogens exert neuroprotective effects against stroke (Finucane et al., 1993, Paganini-Hill, 1995, Wren, 1992). However, recent reports from the Women's Health Initiative studies have indicated detrimental effects of HT on cognition and increased risks of stroke and heart disease (Shumaker et al., 2003, Wassertheil-Smoller et al., 2003). These results are at odds with results of large epidemiological studies that showed protection. Although the latter data are, in part, confounded by a "healthy user bias," much of the inconsistency may be explained by the fact that women in the latter studies initiated HT at the menopausal transition, whereas the WHI trial was conducted in older women who were approximately 12 yr postmenopausal (Harman et al., 2004, Harman et al., 2005, Harman et al., 2005, Harman, 2006). In addition, older trials included women on either unopposed estrogen therapy (ET) or cyclic HT regimens. Further, a variety of other factors confound the issue of the beneficial effects of ET, such as doses of hormones, type of hormones, the age at which HT is administered, and a host of other factors (Singh et al., 2008, Singh, 2007). Therefore, a clearer understanding of the mechanism of estrogen-mediated neuroprotection is important in determining the clinical application of ET. A deeper knowledge of the molecular targets of estrogens will be beneficial in developing estrogen as a potent and effective therapeutic agent against neurodegenerative diseases.

E. Hypothesis

It is well accepted that oxidative stress is involved in a number of neurodegenerative diseases such as AD, PD, and stroke. Experimental and epidemiological studies have demonstrated the neuroprotective effects of estrogens against various toxic insults and also against cognitive decline and memory loss (Paganini-Hill and Henderson, 1996, Green and Simpkins, 2000). Though some of the mechanisms by which estrogens act in the brain have been elucidated, many other targets of estrogen action still needed to be identified. One of the possible targets of estrogens may be integrins and integrin–mediated signaling proteins. Integrins are cell surface receptors that exist in the form of heterodimers and are involved in many of the cellular events like adhesion, migration, proliferation and differentiation. Studies show that integrins are closely involved in the cell survival and cell death signaling pathways (Giancotti and Ruoslahti, 1999). At the same time, there is also evidence that oxidative stress and β-amyloid toxicity causes cell cycle activation and subsequent neuronal cell death via integrin-mediated signaling (Caltagarone, Jing and Bowser, 2007, Frasca et al., 2008). There is evidence that integrins and neural cell adhesion molecules (NCAMs) are involved in the events leading to long-term potentiation (LTP). The purpose of these studies was to determine the interaction of estrogen actions and integrins in neurons.

Based upon published literature, we hypothesized that oxidative stress caused by hydrogen peroxide (H_2O_2) treatment will result in an increase in the protein expression levels of β1-integrin and that 17β-estradiol treatment will attenuate this H_2O_2 induced increase in β1-integrin expression. To address this hypothesis, we proposed the following specific aims.

Specific aim A): To determine if H_2O_2 treatment causes an increase/decrease in the protein expression levels of β1-integrins in a dose-dependent and time-dependent manner.

Specific Aim B): To determine if E2 treatment affects the protein expression levels of β1-integrins in a dose-dependent and time-dependent manner.

Specific aim C): To determine if treatment of E2 along with H_2O_2 has any effect on the protein expression of β1-integrin induced or reduced by H_2O_2 treatment.

The goal of the study was to evaluate the effect of H_2O_2 treatment on the expression of β1-integrins in a murine hippocampal cell line, HT-22. Also, the dose-dependent and time-dependent effects of 17β-estradiol treatment on the expression of β1-integrins in HT-22 cells were determined. As the next step, the effects of 17β- estradiol on the expression of β1-integrins influenced by H_2O_2 treatment were assessed by this study. This study determined the changes in protein expression levels of β1-integrins with H_2O_2 treatment and with 17β-estradiol along with H_2O_2.

From the above experiments, we hypothesized that estrogens might have an effect on the expression of integrins which may be the factor involved in promoting the synaptic connectivity between neurons. With increase in the oxidative stress with H_2O_2 treatment, the neurons seemed to make more connections which correlated with the increase in the protein levels of integrins. The literature also suggested that integrins are localized to synapses and that integrins are involved in the events leading to long-term potentiation (LTP). Therefore, the second hypothesis was that 17β-estradiol treatment may have an effect on the protein expression levels of β1-integrins and may have an important role in promoting the synaptic connectivity between neurons.

Specific Aim D): To determine the effect of E2 treatment on β1-integrins and synaptic proteins in an in vivo study using a rodent model.

The goal of the in vivo study was to determine the effect of 17β-estradiol treatment for a time period that correlates with the peak expression of integrins on the changes that would take place in the synaptic molecular expression of a pre-synaptic protein synaptophysin and two post-synaptic proteins PSD-95 (post-synaptic density-95) and NMDAR1 (N-methyl D-aspartate receptor subunit 1) with the E2 treatment. Ovariectomized female Sprague-Dawley rats which are three months old were used for the study.

F. Rationale for the study

The study was focused on the induction of expression of β1-integrins, in particular, because these comprise the major class of integrins and most α subunits form heterodimers with β1 subunit. Also, integrins of β1 family are key mediators of neural development and may play a role in synaptic plasticity and neural disease. The proposed research study is directed to determine if integrins are one of the molecular targets of estrogens as this could be beneficial in developing estrogen as a potent and effective therapeutic agent against neurodegenerative diseases.

CHAPTER II

MATERIALS AND METHODS

A. Cell culture

HT-22 cells provided by Dr. David R Schubert, (Salk Institute, San Diego, CA) are immortalized, murine hippocampal cell line. The cells were maintained in Dulbecco's modified eagle's media (DMEM) (GIBCO, Gaithersburg, PA) supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) and 100 U/ml penicillin, 100μg/ml streptomycin at 37°C in 5% CO2 and 95% air. Cells were plated into 100mm or 150mm culture dishes and used for Western analysis when they reach 70% confluency.

B. Chemicals

17β-estradiol was purchased from Steraloids, Inc. (Wilton, NH) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted to appropriate concentration in culture media. Hydrogen peroxide (H_2O_2, 30%) was purchased from Mallinckrodt baker, Inc. (Paris, KY). β1-Integrin(C-terminus) rabbit polyclonal IgG antibody, β1-Integrin(N-terminus) goat polyclonal IgG antibody, PSD-95 goat polyclonal antibody, Synaptophysin mouse monoclonal antibody and β-actin mouse monoclonal primary antibody were purchased from Santa Cruz biotechnology, Inc. (Santa Cruz, CA). NMDAR1 rabbit

polyclonal primary antibody was purchased from Cell signaling Technology, Inc. (Cell Signaling Technology, MA).

C. Treatments

HT-22 cells were treated with various concentrations of H_2O_2 ranging from 0 to 700μM and through a period of 20 hr for dose-dependent and time-dependent studies. 17β-estradiol was used at various concentrations ranging from 0.01μM to 10μM. For combined study HT22 cells were treated with both H_2O_2 and 17β-estradiol simultaneously.

D. Preparation of whole cell lysates

The whole cell lysates of treated HT22 cells from culture dishes were prepared using RIPA lysis buffer (5mM NaCl, 0.5mM Tris pH 7.2, 10% Triton X-100, 10% deoxycholate, 100 mM EDTA, 50 mM PMSF, 100mM Na-O-Vanadate, Protease inhibitor cocktail). The cell pellet was washed in PBS buffer and centrifuged for 3 min at 4000rpm and then the PBS was aspirated out. About 100 to 150μl of lysis buffer was added, vortexed and left on ice for 30 min. Then the samples were sonicated for about 30 seconds on ice and centrifuged at 5000 rpm for 5 min. The supernatant was collected from the samples for use in Western blot analysis. Protein concentration of the samples was determined using Bio-rad protein assay reagent.

E. Western Blot

Protein from whole cell lysates (20μg) was separated by SDS-polyacrylamide gel electrophoresis (8% gel) and transferred to immunobilon–P polyvinylidene difluoride

(Millipore Corp., Bedford, MA) membrane. Membranes were gently rinsed in Tris-buffered saline (10mM Tri-base, pH 8.0, 100mM NaCl) containing 0.2% Tween 20 and blocked with 5% dry milk solution for 2 hrs at room temperature. The membranes were incubated with β1-integrin primary antibody (1:200) and β-actin primary antibody (1:1000) overnight at $4^{o}C$. The membranes were then washed thrice and incubated with appropriate secondary antibody for 2 hrs at room temperature. The protein bands were detected using enhanced chemiluminescence (ECL; Pierce Biotechnology, Rockford, IL). ECL results were quantified using UVP Bioimaging system (Up-Land, CA).

F. Statistical analysis

Statistical significance was determined by one-way ANOVA followed by a Tukey's multiple comparison test. $p<0.05$ or $p<0.01$ was considered significant between two different groups. Each set of data represented two or more independent experiments. The data was represented as mean +/-SEM.

G. In vivo study

a) Animals

Female Sprague–Dawley (3mo. old) were purchased from Charles River Labs (Wilmington, MA) and maintained in pairs in temperature-controlled rooms (22–25 °C) with 12-h light–dark cycles. All rats had free access to laboratory chow and tap water. Bilateral ovariectomy was performed 2 weeks before E2 treatment under anesthesia with intraperitoneal (i.p.) injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). A small cut was made through

skin and muscle, and ovaries were externalized and removed. The ovariectomy was done to avoid fluctuations of estrogen, which are neuroprotective. All animal procedures were reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee, and adhered to follow current standards.

b) Treatment

For E2 treatment, E2 was dissolved in corn oil and was administered at the dose of 100 μg/kg body weight via subcutaneous injection. Corn oil alone was administered for the vehicle control animals. A 24hr sampling time was used for this study.

c) Collection of brain tissue

After the treatment, all the animals were euthanized and their brains harvested. The hippocampus and cortex were dissected out and lysed for Western blot analysis.

d) Preparation of brain tissue lysates

The brain tissue was homogenized using lysis buffer containing 50mM MOPS(x), 100mM KCl, 2% TritonX-100, 1mM EDTA, 10mM NaF, 1% NP-40, 10mM Na_3VO_4 , 50mM PMSF, Protease Inhibitor cocktail. After homogenization, the samples were left on ice in the lysis buffer for at least 1 hour. Then the samples were sonicated on ice for about 20 min and centrifuged at 15,000 rpm for 20 mins. The supernatant was collected from the samples for use in Western blot analysis. Protein concentration of the samples was determined using Bio-rad protein assay reagent.

e) Western blot

Protein from tissue lysates was separated by SDS-polyacrylamide gel electrophoresis and transferred to immunobilon–P polyvinylidene difluoride (Millipore Corp., Bedford, MA) membrane. Membranes were gently rinsed in Tris-buffered saline (10mM Tri-base, pH 8.0, 100mM NaCl) containing 0.2% Tween 20 and blocked with 5% dry milk solution for 2 hrs at room temperature. The membranes were incubated with β1-Integrin primary antibody (1:200), Synaptophysin primary antibody (1:1000), PSD-95 primary antibody (1:200), NMDAR1 primary antibody (1:200) and β-actin primary antibody (1:1000) overnight at 4°C. The membranes were then washed thrice and incubated with appropriate secondary antibody for 2 hrs at room temperature. The protein bands were detected using enhanced chemiluminescence (ECL; Pierce Biotechnology, Rockford, IL). ECL results were quantified using UVP Bioimaging system (Up-Land, CA).

f) Statistical analysis

Statistical significance was calculated using Student's *t* test to determine whether compared groups are distinct. The compared groups were considered significantly distinct if $p<0.05$. The data was presented as mean+/- SEM.

CHAPTER III

RESULTS - *IN VITRO* STUDY

A) Concentration and time-dependent effects of H_2O_2 treatment on β1- integrin expression in HT-22 cells

To determine the concentration-dependent effect of H_2O_2 treatment on β1- integrin expression, HT-22 cells were cultured in 100mm dishes until they reached 60%-70% confluency. Then the cells were treated with various concentrations of H_2O_2 ranging from 0 μM to 700 μM for a period of 20 hrs. The cells were harvested and the lysates were prepared from all the treatment groups for Western blot analysis of β1- integrin protein. For densitometry analysis, the band intensities were taken and intensity of each band was normalized to that of the β-actin band.

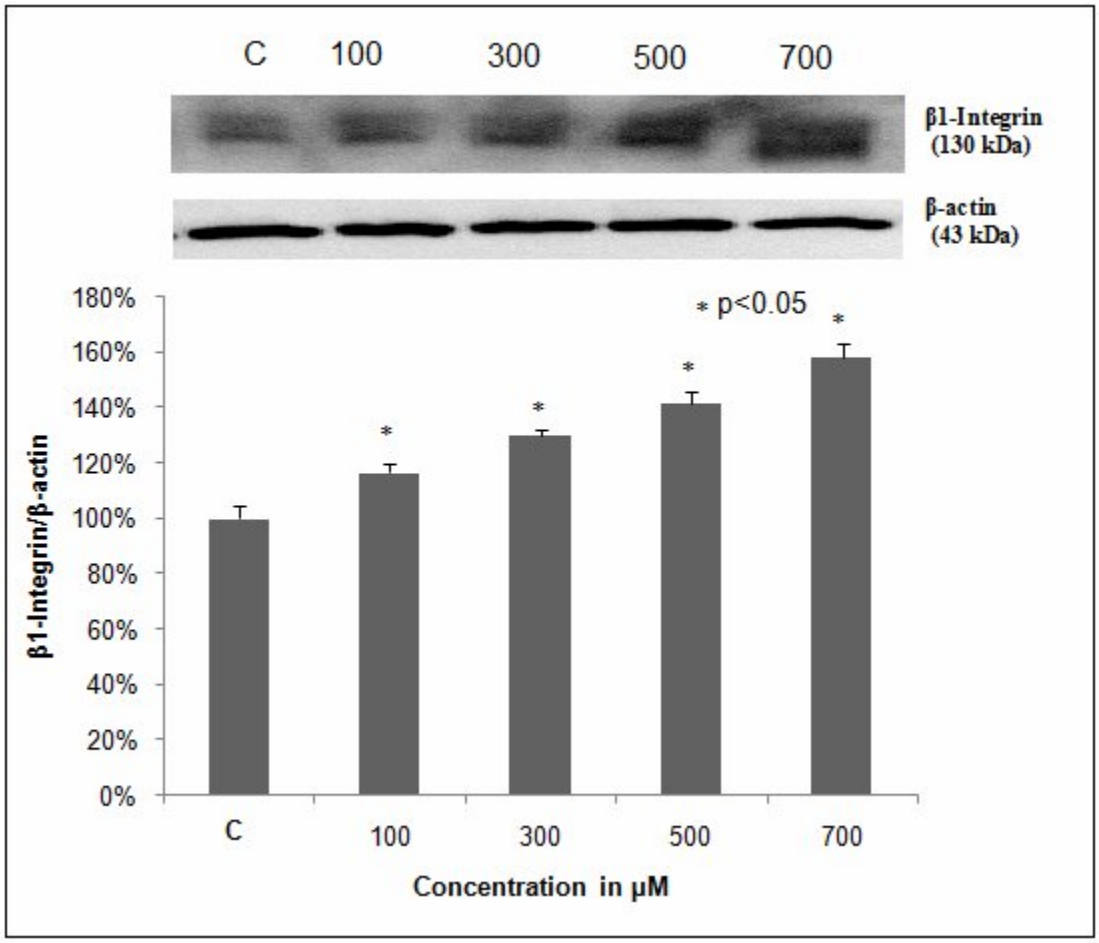

Figure 5: Concentration-dependent effect of H_2O_2 treatment on β1-integrin expression.

The relative levels of β1-integrin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of control. The data is presented as mean+/- SEM from three independent experiments. *, *p<0.05* versus control group; 0 group is considered the control group.

This study addressed specific aim A) which is to determine if hydrogen-peroxide treatment causes an increase/decrease in the expression of β1-integrins in a concentration-dependent manner. Figure 5 demonstrates the concentration-dependent increase in the expression of β1-integrin protein expression. At 700μM H_2O_2 treatment, a peak in the expression with a 60% increase was observed compared to the control levels.

The time-course effect of H_2O_2 treatment on β1- integrin expression was determined. HT-22 cells were cultured in 100mm dishes until they reached 60%-70% confluency. Then the cells were treated with 700 μM concentration of H_2O_2, since at this concentration the expression of β1- integrins was found to be the highest. The cell lysates were collected at different time points ranging from 1hr to 16hr. Western blot analysis was performed to measure the protein expression levels of β1-integrin as a function of time.

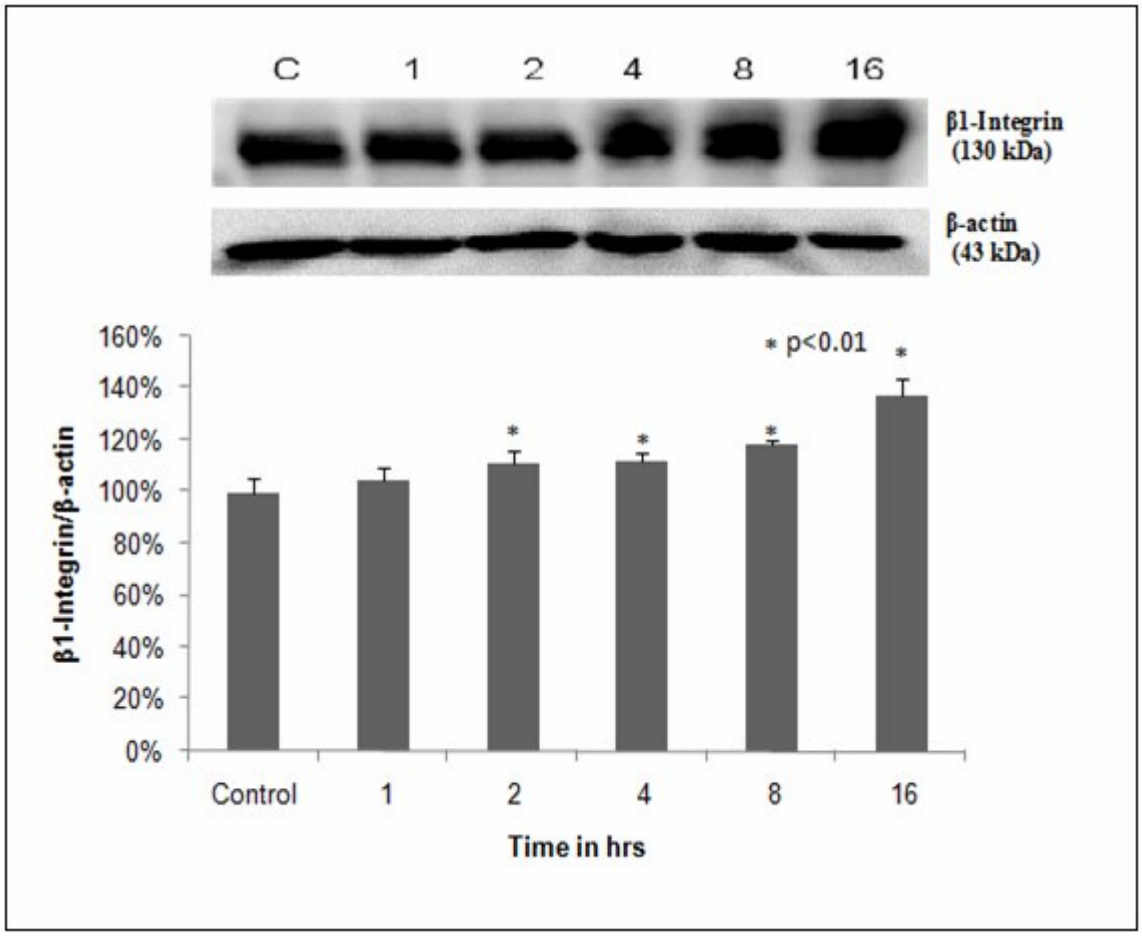

Figure 6: Time-course of H_2O_2 treatment on β1- integrin expression

The relative levels of β1-integrin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of control. The data is presented as mean+/- SEM from three independent experiments. *, *p<0.01* versus control group

This study addressed specific aim A) which is to determine if hydrogen-peroxide treatment causes an increase/decrease in the expression of β1-integrins in a time-dependent manner. The intention was to determine if the increase in the expression of β1-integrins after H_2O_2 treatment followed a time course. Figure 6 shows an increase in β1-integrin expression at 2hr post-treatment and continued to increase throughout the course of the treatment. At 16hr post-treatment, a 40% increase in protein level of β1-integrin was observed. Not only is the protein expression of β1-integrins concentration-dependent, it was also time-dependent.

B) Concentration and time-dependent effects of E2 treatment on β1-integrin expression in HT-22 cells

To determine the concentration-dependent effect of E2 treatment on β1-integrin expression, HT-22 cells were cultured in 100mm dishes until they reached 60%-70% confluency. Then the cells were treated with various concentrations of E2 ranging from 0.01 μM to 10 μM for a period of 24 hrs. The cell lysates were prepared from all the treatment groups and Western blot analysis was performed to measure the β1-integrin expression. For densitometry analysis, the band intensities were taken and intensity of each band was normalized to that of the β-actin band.

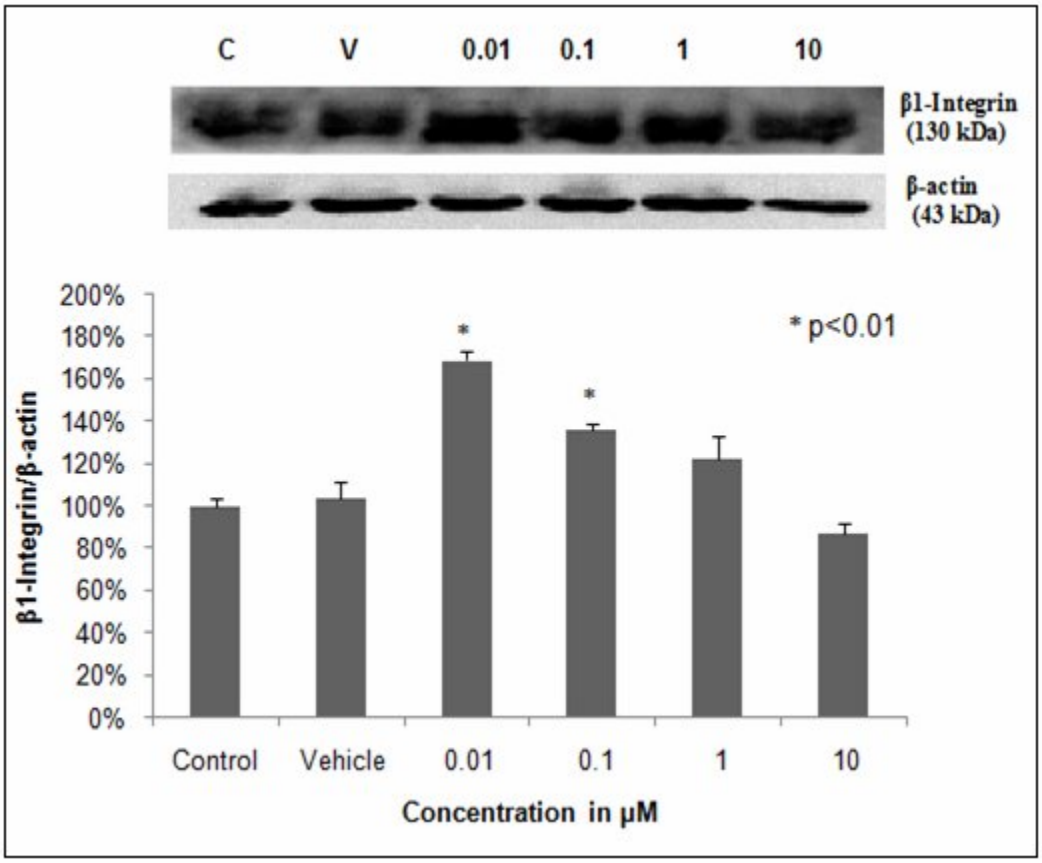

Figure 7: Concentration-dependent effects of E2 treatment on the expression of β1-integrin expression

The relative levels of β1-integrin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of control. The data is presented as mean+/- SEM from three independent experiments. *, *p<0.01* versus control group; vehicle is DMSO.

This study addressed specific aim B) which is to determine if E2 treatment effects the expression of β1-integrins in a concentration-dependent manner. Figure 7 demonstrates an increase in the protein level of β1-integrins with 10nM and 100nM E2 compared to the control group. A peak in the expression of β1-integrin was observed for 10nM E2 treated group with a 65% increase compared to that of the control. This study suggested that physiologically relevant concentrations of E2 or low doses of E2 have a significant effect on the expression of β1-integrins than the pharmacological concentrations.

To determine the time course effect of E2 treatment on β1- integrin expression, HT-22 cells were cultured in 100mm dishes until they reached 60%-70% confluency. Then the cells were treated with 10nM concentration of E2, since at this concentration the expression of β1- integrins was found to be higher from the concentration-response study. The cell lysates were collected at different time points ranging from 1hr through 24hr. and Western blot analysis was performed to measure the protein expression levels of β1- integrin at all time points.

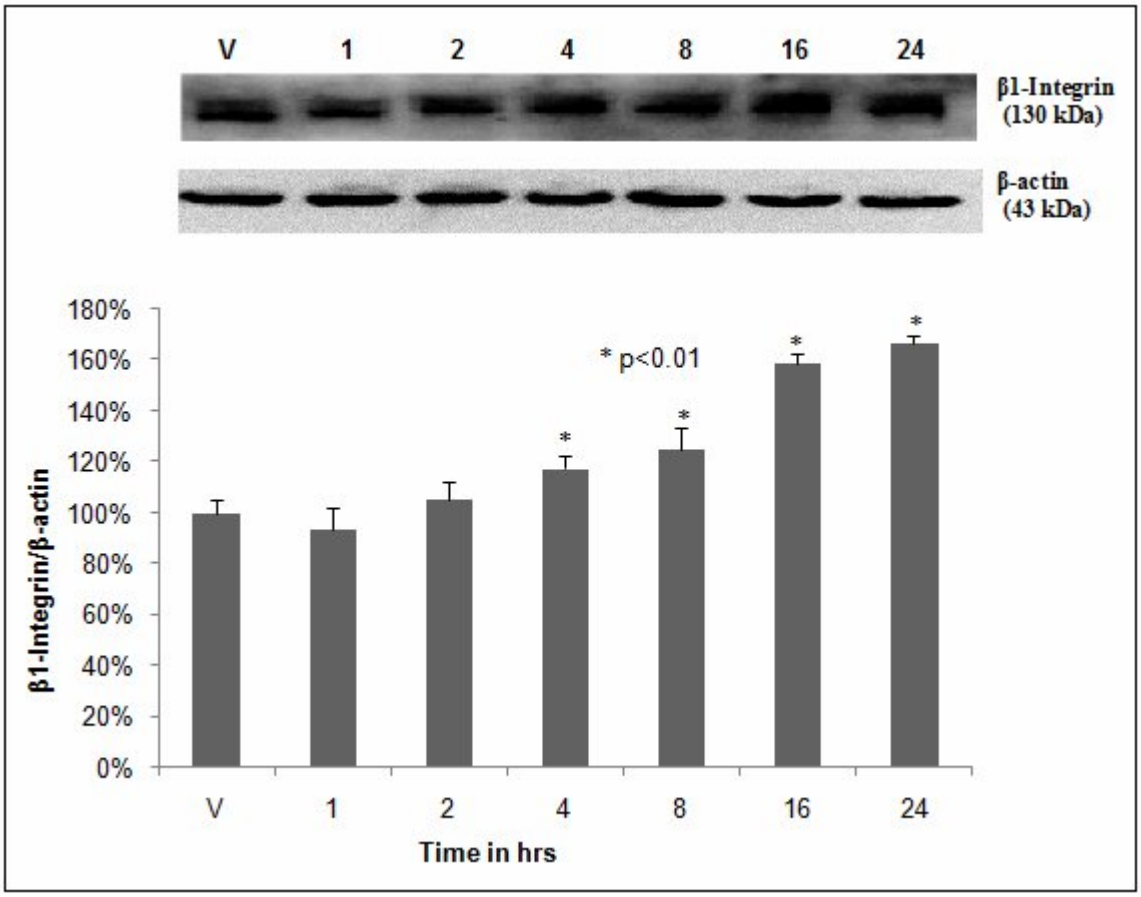

Figure 8: Time course effect of E2 treatment on β1- integrin expression.

The relative levels of β1-integrin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM from three independent experiments. *, *p<0.01* versus vehicle treated group; vehicle is DMSO.

This study addressed specific aim B) which is to determine if E2 treatment effects the expression of β1-integrins in a time-dependent manner. An increase in β1-integrin expression was observed at 4hr post-treatment and continued to increase throughout the time course. At 24hr time point there was a 60% increase in the protein level of β1-integrin compared to the vehicle group.

C) Effect of E2 treatment along with H_2O_2 treatment on the protein expression levels of β1-integrin.

To determine the effect of E2 on H_2O_2 induced β1-integrin expression, HT-22 cells were cultured in 100mm dishes until they reached 60%-70% confluency. The cells were treated with 10 μM 17β-estradiol simultaneously with 700 μM concentration of H_2O_2 for a period of 16 hrs. The cell lysates were prepared from all the treatment groups and Western blot analysis was performed to measure the protein expression levels of β1- integrin. For densitometry analysis, the band intensities were taken and intensity of each band was normalized to that of the β-actin band.

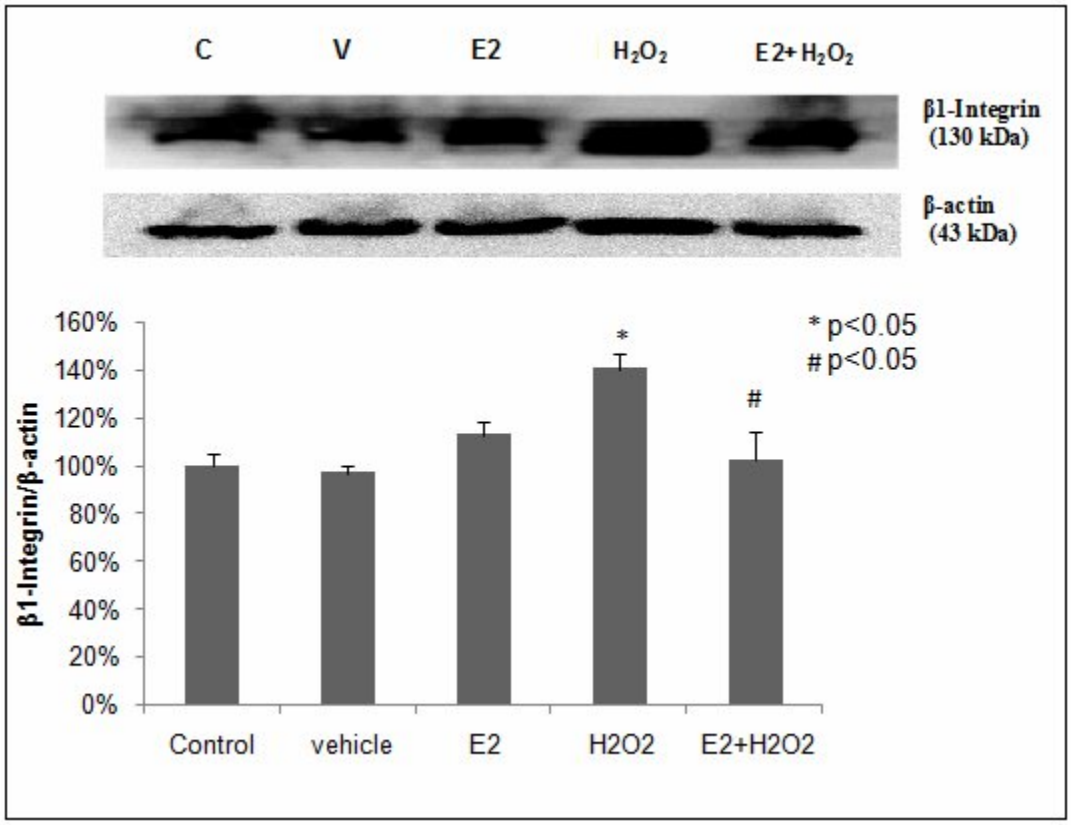

Figure 9: Effect of E2 treatment on H_2O_2 induced β1-integrin expression

The relative levels of β1-integrin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of control. The data is presented as mean+/- SEM from two independent experiments. *, $p<0.05$ versus control group; #, $p<0.05$ versus H_2O_2 treated group; vehicle is DMSO.

This study addressed specific aim C) which is to determine if E2 treatment effects the expression of β1-integrins induced by H_2O_2. For the co-treatment study, HT-22 cells were treated simultaneously with 10μM E2 and 700μM H_2O_2 since the results from specific aims A and B have shown a peak in the expression of β1-integrins with 700μM concentration of H_2O_2 and no change in the expression of β1-integrins with 10μM of E2 respectively in HT-22 cells. In this study, H_2O_2 alone caused an increase in the expression of β1-integrin by about 40% and when E2 was present with H_2O_2, the protein level of β1-integrin was not different than the control group as shown in Figure 7.

CHAPTER IV

RESULTS – *IN VIVO* STUDY

D) Effect of E2 pretreatment on β1-integrins and synaptic proteins in an in vivo study using a rodent model.

To determine the effect of E2 treatment on the expression of β1- integrins and synaptic proteins, 10 female Sprague-Dawley rats were ovariectomized and divided into two groups, one group for vehicle treatment and one for E2 treatment. E2 was dissolved in corn oil and was administered at the dose of 100 μg/kg body weight via subcutaneous injection. Corn oil alone was administered for the control animals. A 24hr sampling time was used for this study. After the treatment, all the animals were euthanized and their brains harvested. The hippocampus and cortex were dissected out and lysed for further use in Western blot analysis. Western blots were performed to measure the protein expression levels of β1- integrin, synaptophysin, PSD-95 and NMDAR1 in all the groups. For densitometry analysis, the band intensities were taken and each band was normalized to that of the β-actin band.

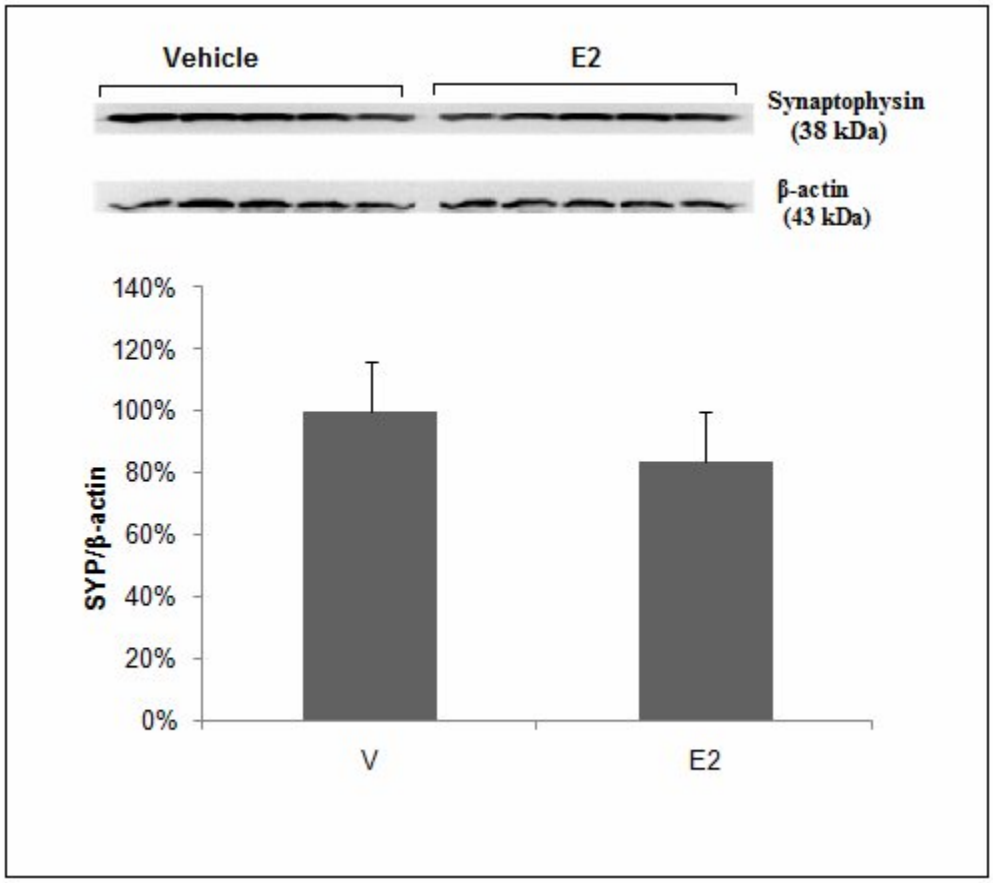

Figure 10: Effect of E2 treatment on synaptophysin expression in hippocampus.

The relative levels of synaptophysin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM (*n*=5).

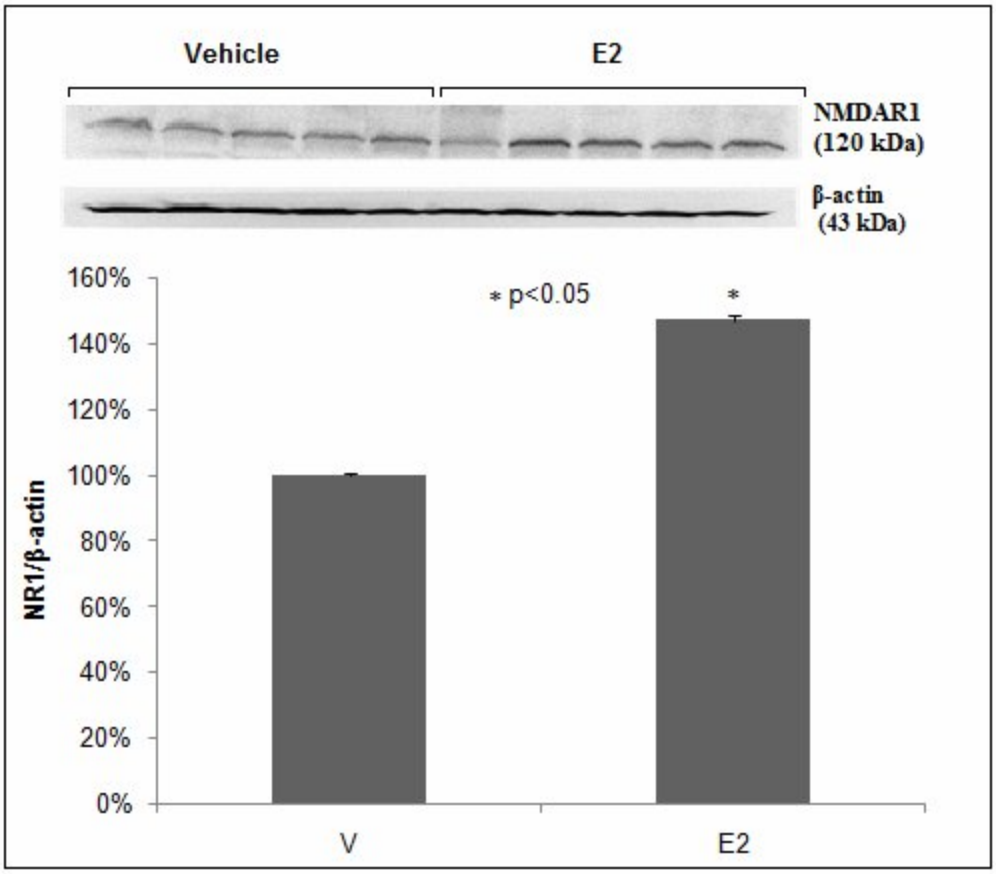

Figure 11: Effect of E2 treatment on NMDAR1 expression in hippocampus.

The relative levels of NMDAR1 expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM (*n*=5). *, *p<0.05* versus vehicle treated group

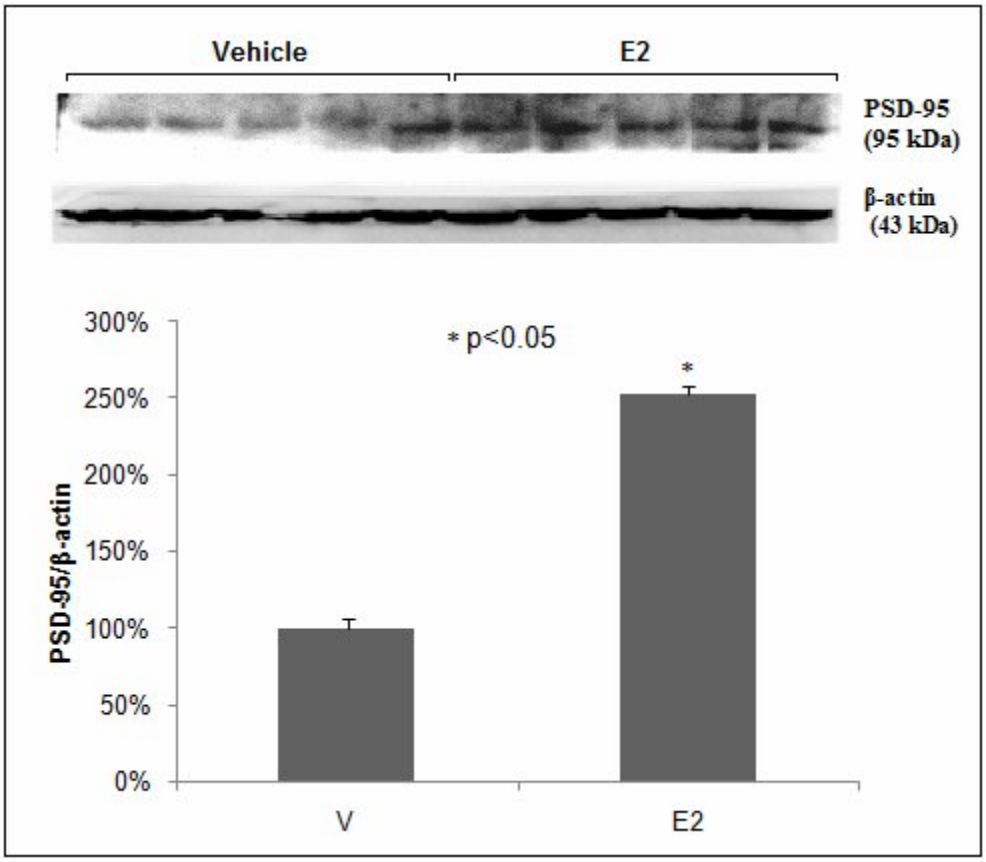

Figure 12: Effect of E2 treatment on PSD-95 expression in hippocampus.

The relative levels of PSD-95 expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM (*n*=5). *, *p<0.05* versus vehicle treated group

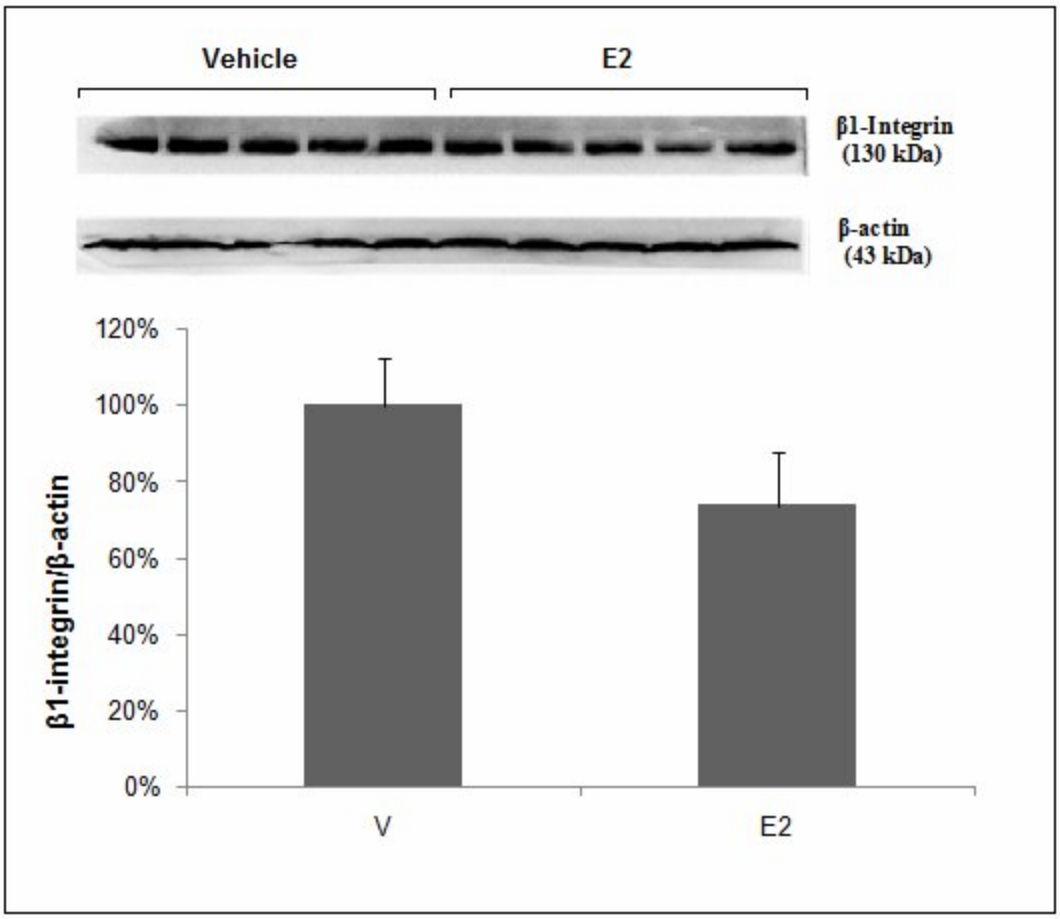

Figure 13: Effect of E2 treatment on β1-integrin expression in hippocampus.

The relative levels of β1-integrin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM (*n*=5).

Figure 10 shows the effect of E2 treatment on the protein expression levels of Synaptophysin in hippocampus, which is a pre-synaptic protein. There were no significant differences observed between the vehicle treated group and E2 treated group suggesting that E2 did not affect the expression of synaptophysin in this in vivo study.

Figure 11 demonstrates the effect of E2 treatment on the protein expression levels of NMDAR1 in hippocampus, which is a protein localized to the post-synaptic membrane or post-synaptic density. Within the E2 treated group a 47% increase in the protein level of NMDAR1 was observed compared to that of the vehicle treated group.

Figure 12 demonstrates the effect of E2 treatment on the protein expression levels of PSD-95 in hippocampus, which is a fundamental structural protein found in the post-synaptic density. There was a significant increase in PSD-95 protein levels with a 150% rise in E2 treated group compared to that of the vehicle group.

Figure 13 shows the effect of E2 treatment on the protein expression levels of β1-integrin in hippocampus, which is believed to be localized to the synaptic architecture. Although, there appears to be a slight decrease in the expression level of integrin in E2 treated group, the differences between the vehicle treated group and the E2 group were not found to be significant indicating that E2 did not have an effect on β1-integrin expression in this in vivo study.

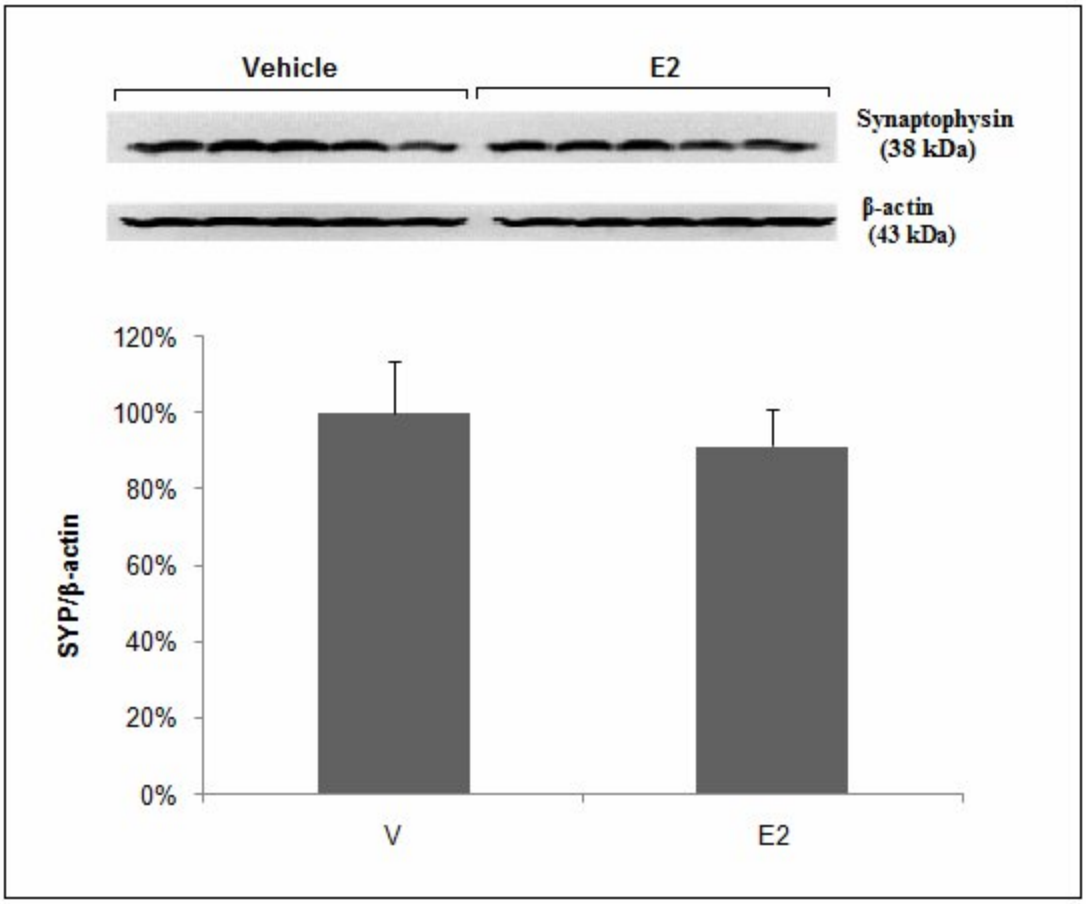

Figure 14: Effect of E2 treatment on Synaptophysin expression in cortex.

The relative levels of synaptophysin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM (*n*=5).

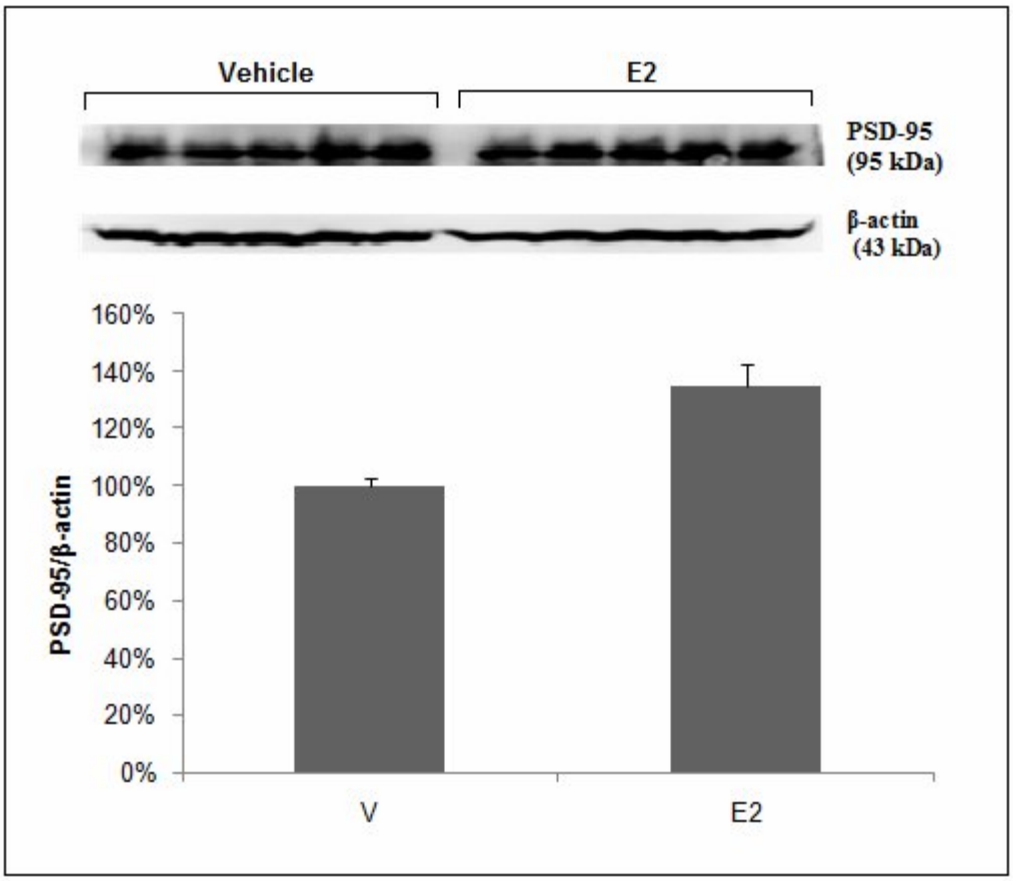

Figure 15: Effect of E2 treatment on PSD-95 expression in cortex.

The relative levels of PSD-95 expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM (*n*=5).

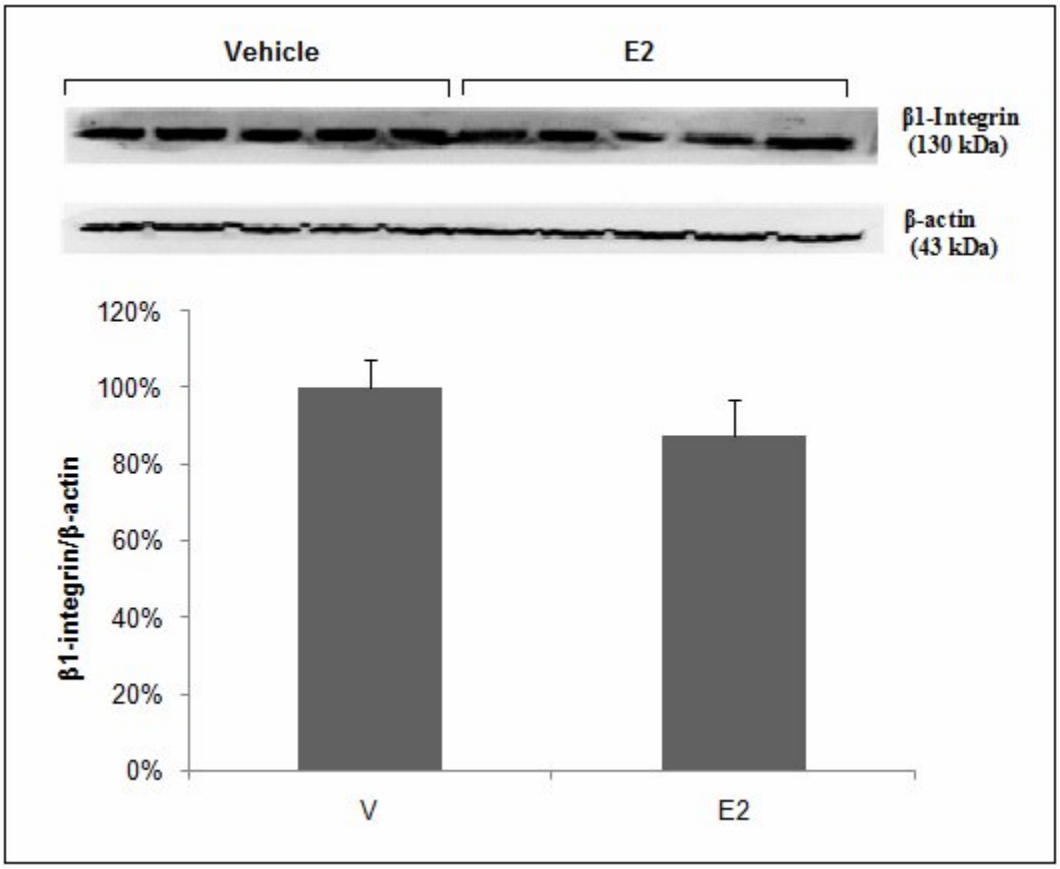

Figure 16: Effect of E2 treatment on β1-integrin expression in cortex.

The relative levels of β1-integrin expression were measured by densitometry analysis of immunoblots. The data was normalized to β-actin to ensure equal protein loading, and the normalized data was expressed as % of vehicle. The data is presented as mean+/- SEM (*n*=5).

Figures 14, 15 and 16 depicted the effects of E2 treatment on the protein expression levels of synaptophysin, PSD-95 and β1-integrin in the cortex section of the brain. There were no significant differences observed in any of the above mentioned protein levels between the vehicle treated group and E2 treated group suggesting that E2 did not affect the expression of synaptophysin, PSD-95 and β1-integrin in this in vivo study.

In summary, H_2O_2 treatment caused an increase in the β1-integrin expression in a concentration and time-dependent manner in vitro. E2 treatment alone also caused an increase in the protein expression level of β1-integrin with physiological concentrations of E2 and a time-dependent manner increase was observed with 10nM concentration of E2 in vitro. E2 treatment along with H_2O_2 caused a suppression of β1-integrin expression induced by H_2O_2 treatment in vitro. In the in vivo study, E2 treatment caused an increase in the protein expression levels of the two post-synaptic proteins observed, which are NMDAR1 and PSD-95 in the hippocampus while there was no significant effect observed in synaptophysin and β1-integrin levels in hippocampus. E2 treatment also did not produce a significant effect on the expression levels of any of the above mentioned proteins in the cortex compared to the levels of vehicle treated group.

CHAPTER V

DISCUSSION

In the present study, hydrogen-peroxide was used as a pro-oxidant to induce oxidative stress in HT-22 cells and the effect on integrin expression was observed. There was a concentration and time-dependent increase in β1-integrin expression observed with increasing concentrations of H_2O_2. This effect can be attributed to the compensatory response that the cells were exhibiting in order to survive from the oxidative damage induced by H_2O_2. Increase in the expression of integrins might be helping the cells to preserve the cell-cell as well as cell-matrix interactions in an attempt to maintain cell shape and integrity. Another possibility is that with an increase in oxidative stress, the cells are entering the cell cycle and they are probably being committed to apoptosis and the increase in β1-integrin expression with H_2O_2 treatment is probably acting as a death signal in this scenario.

The estrogen alone study was done using various concentrations of E2 ranging from nanomolar to micromolar doses in HT-22 cells. There was a significant increase in the expression of β1-integrins observed with 10nM and 100nM concentrations of E2. The study indicates that physiologically relevant concentrations of E2 caused an increase in the expression of integrins while pharmacological concentrations did not have an effect on the expression of integrins. The mechanism involved in this E2 mediated effect is yet to be determined.

A number of studies have shown the involvement of hormones in altering the integrin synthesis, expression and function. Treatment of human umbilical vein endothelial cells with 2 ng/ml 17β-estradiol induced an increase in the surface expression of β1-integrins promoting cellular adhesion to extracellular matrix proteins (Cid et al., 1999). A two-fold increase in β1-integrin mRNA levels was also seen with E2 treatment (Cid et al., 1999). Enhancement of integrin expression and function by estrogens not only resulted in accelerated cell attachment and spreading in the endothelial cell line but also promoted integrin-mediated signaling by an increase in tyrosine phosphorylation of FAK (Focal adhesion kinase) (Cid et al., 1999). 17β-estradiol was shown to significantly stimulate the expression of β1-integrins in human mammary epithelial cells (MCF-7 cells) when compared to phytoestrogens (Nebe et al., 2006). *In vivo*, it was shown that α5β1 integrin levels increase from puberty to early pregnancy and decrease at late pregnancy and lactation during mouse mammary gland development. Level of α5β1 integrins was shown to be significantly down-regulated following ovariectomy and up-regulated rapidly after estrogen and progesterone treatments in both mammary epithelial cells and myoepithelial cells (Haslam and Woodward, 2001). The above mentioned review suggests that a coordinated regulation of ovarian hormone induced changes in ECM/integrin expression is necessary for normal breast tissue development and for preventing breast cancer growth and progression. Therefore, a balance in estrogen regulation of integrin-mediated signaling is necessary for proper development of tissue architecture and integrin-dependent functions. Moreover, estrogens and integrins have been shown to share common signaling pathways, both are involved in the regulation of growth factor-mediated signaling pathways like Ras/Raf/ERK (MAPK) pathway and the PI-3K/Akt pathway involved in the regulation of cell survival and death. The role of estrogen in the processes leading to synaptic plasticity is very well

documented (Spencer et al., 2008). The literature suggests that integrins also are involved in the events leading to synaptic plasticity. Therefore, there is the possibility that there is cross-talk between the signaling pathways mediated by these two important molecules in normal physiological state as well as pathophysiological conditions. One such example comes from a review focusing on the regulatory role of estrogens in modulating the vascular endothelial cell responses (Cid, Schnaper and Kleinman, 2002). The following is a diagrammatic representation of how estrogens regulate cross-talk between integrins and growth factors through genomic and non-genomic mechanisms. The production and release of FGF-2 (Fibrinogen growth factor-2) is mediated by estrogen receptor, non-genomic, PKC dependent mechanism and FGF-2 is able to upregulate endothelial cell integrins.

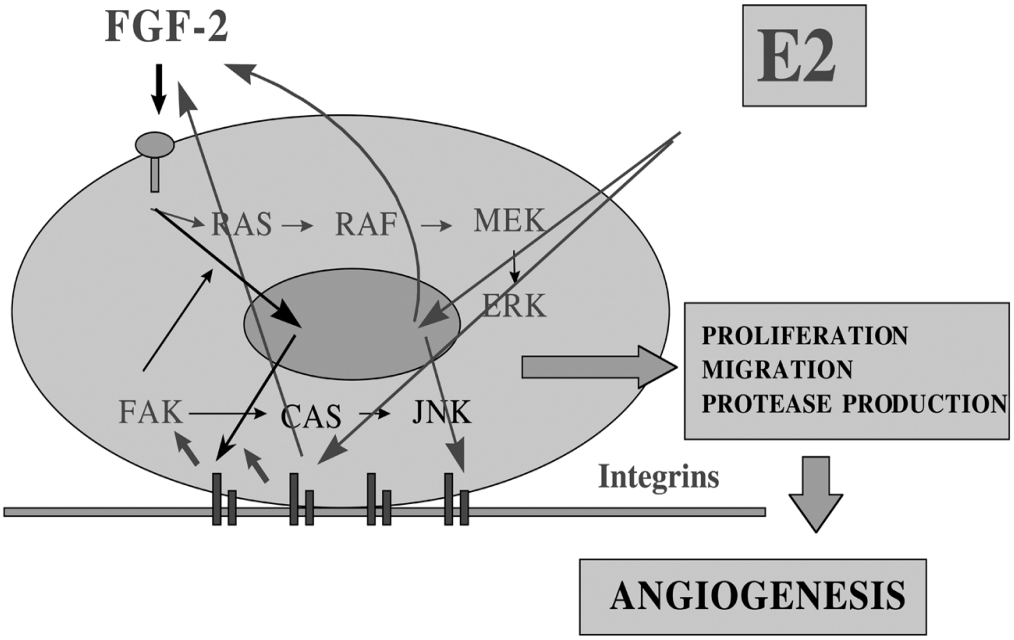

Fig 17: Estrogen regulates cross-talk between integrins and growth factors at different levels through genomic and non-genomic mechanisms (Cid, Schnaper and Kleinman, 2002).

While integrins and integrin-mediated signaling are involved in cell proliferation, growth and survival which are important for maintaining normal physiological functions, the same are also involved in pathological conditions like cancer metastasis, inflammation etc. It is also known that oxidative damage activates integrin-mediated signaling pathways, pushing the cell to enter cell cycle and ultimately causing neuronal cell death (Caltagarone, Jing and Bowser, 2007). Therefore, there has to be a balance between integrin expression and integrin activation of signaling for maintenance of normal cellular functions.

In in vitro studies, neuroprotective concentrations of E2 have been indicated to range from low nanomolar to high micromolar concentrations. In cell culture studies, a variety of factors such as cell type, cell density, culture conditions, type of insult and severity of insult etc. contribute to the wide range of protective concentrations of E2. In our laboratory, a pharmacological concentration of E2 which is 10μM was established as a potent neuroprotective concentration in vitro against many oxidative insults including H_2O_2. Estrogen was also shown to have direct antioxidant properties due to the presence of the phenolic-A ring in the structure. While neuroprotection by E2 can be seen even with significantly lower concentrations, antioxidant effects of estrogens require μM concentrations (Green and Simpkins, 2000). Since the insult being used for this study is H_2O_2, which is a major ROS and the concentration used is 700μM which is a very toxic concentration producing 60-70% cell death (data not shown), for the co-treatment study it was found to be appropriate to use 10μM concentration of E2 to determine its effect on the induction of integrins by H_2O_2. Therefore, for the co-treatment study, HT-22 cells were treated simultaneously with 10μM E2 and 700μM H_2O_2. In this study, H_2O_2 alone caused an increase in the expression of β1-integrin by about 40% and when E2 was present

with H_2O_2, the protein level of β1-integrin was not different than the control group as shown in Figure 9.

When HT-22 cells were treated with E2 along with H_2O_2, the expression of β1-integrins was reduced to that of the control group. Since the concentration of E2 used for the co-treatment study was in micromolar concentration, a possible explanation for this effect is that, E2, by means of its antioxidant ability might be rendering the H_2O_2 inactive before it even causes any oxidative damage to the cells and thereby, making the increase in integrin expression unnecessary. The possibility of estrogen itself directly causing a decrease in the expression of β1-integrins is not likely to be occurring because with this concentration of E2 (10μM) there was no significant effect observed on the expression of β1-integrins. One way to confirm the antioxidant role played by E2 in causing the decrease of integrin expression is to use another compound known to have antioxidant activity and to determine if this compound can produce the same effect as E2. For future studies, it would be interesting to test this aspect of antioxidant role as this was not tested in this particular study. One more possible explanation for this effect is that during oxidative stress, E2 might be affecting the expression of integrins indirectly through non-genomic mechanisms such as regulating signaling pathways involved in cell survival. The mechanisms involved in the effect of E2 on integrin expression needs to be studied further for a better understanding of the role of E2 during oxidative stress conditions.

Many studies have reported the involvement of integrins in the events leading to synaptic plasticity and memory formation. The critical role of integrins in mediating long-term potentiation (LTP) is evidenced by pharmacological, genetic as well as behavioral studies. RGD containing peptides, function blocking antibodies, disintegrins etc. which are all integrin antagonists blocked the consolidation of LTP at hippocampal Schaffer collateral synapses (Bahr

et al., 1997, Staubli, Chun and Lynch, 1998). Mice with forebrain specific loss of β1-integrin exhibited impaired LTP (Huang et al., 2006, Chan et al., 2006). Behavioral studies on the same mice (forebrain-specific β1-integrin knock outs) demonstrated impairment in hippocampus-dependent working memory but did well on other hippocampus-dependent tasks such as spatial memory and conditioned fear (Chan et al., 2006). However, the differential role of β1-integrins in affecting both the tasks is still unclear.

As the next step, an in vivo study was done to test the effect of E2 treatment on integrins in vivo and also to see if a positive effect on integrins could have an impact on the synaptic proteins. In our study, there were no significant changes in integrins observed with E2 treatment, although, there appears to be a slight decrease in the protein levels of integrins within E2 treated group. On the other hand, E2 treatment has caused an increase in the expression of synaptic proteins, particularly, the post-synaptic proteins PSD-95 and NMDAR1, although significant increases were not noticed in the cortical tissues which were E2 treated. Also, the pre-synaptic protein synaptophysin expression was unaffected by the E2 treatment in both hippocampus as well as cortex. From this study, it is very hard to conclude if integrins or integrin-mediated signaling is involved in altering the expression of synaptic proteins because there are no significant differences observed in β1-integrin protein levels with E2 treatment. Even though, we expected an increase in the expression of β1-integrin protein based on the results from in vitro studies, there was no significant effect of E2 observed on the protein *in vivo*. The reason for this might be that the concentration of E2 used in the *in vivo* study falls down to either 1 or 2 nM by the end of the sampling time 24hrs because of the rapid metabolism of E2. These concentrations are much lower when compared to the concentration of E2 at which the effect on β1-integrin protein expression was observed *in vitro*. Another possibility is that the cell line used for the *in*

vitro study is the HT-22 cell line which is a transformed hippocampal cell line and do not represent a true neuron. The effects of E2 on HT-22 cell physiology might be completely different when compared to those on the whole brain.

Few studies have demonstrated the involvement of integrins in the pathophysiology of stroke. Integrin αvβ3 is selectively upregulated during focal cerebral ischemia and inhibition of this receptor with selective integrin αvß3 inhibitor was found to improve outcomes in the stroke model which is associated with improved blood-brain barrier integrity and decreased activity of vascular endothelial growth factor (VEGF) (Shimamura et al., 2006b, Shimamura et al., 2006a).

There are a number of studies that have reported the influence of estradiol treatment on synaptic protein expression which, in turn, have an important role in modulating synaptic architecture and thereby, affecting the events leading to long-term potentiation (LTP) or learning and memory. It was shown that across the estrous cycle, 17β-estradiol plays an important role in the cyclic increases in dendritic spine density and synaptogenesis in the CA1 region of the rat hippocampus (Woolley and McEwen, 1992). When circulating estrogens are at high concentrations at proestrous, and on exogenous administration of E2 to ovariectomized female rats in vivo and to primary cultured neurons in vitro, there is a significant increase in the dendritic spine density in the CA1 neurons of the hippocampus. Such an increase in spine density by E2 stimulation leads to enhancement of long-term potentiation sensitivity and improvement in performance in learning and memory tasks (McEwen et al., 2001). PSD-95 is a 95kDa scaffolding structural protein located in the PSD (post-synaptic density) containing multiple domains to anchor and associate glutamate receptors with other structural proteins in the PSD. ER-α receptor was also shown to be localized at the PSD of hippocampal pyramidal neurons which indicates a means by which estrogen regulates synaptogenesis (McEwen et al., 2001,

Woolley, 1999). In vitro, 17β-estradiol treatment of differentiated neuroblastoma cells was shown to lead to the rapid phosphorylation of Akt or PKB (protein kinase B) which causes hyperphosphorylation of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) that relieves its translational repression (Akama and McEwen, 2003). This ultimately led to the translation of PSD-95 causing an increase in the protein synthesis of PSD-95, although it did not cause a significant increase in the mRNA levels of PSD-95 (Akama and McEwen, 2003). Another group demonstrated a similar effect, which is an increase of expression of PSD-95 with estradiol treatment in vivo. In this study, the group has demonstrated an increase in the protein expression of PSD-95 and AMPA receptor subunit GluR1 with estradiol and ER-β agonist treatment of OVX rats which associates ER-β receptor activation to the effect on synaptic proteins and hippocampal synaptic plasticity (Liu et al., 2008). Consistent with these studies, our study has also showed an increase in the protein expression of PSD-95 in the hippocampal tissue with E2 treatment after ovariectomy.

Another important observation that was made from our study was that with E2 treatment, there was a significant increase in the protein expression of NMDAR1 subunit. Many studies have shown that NMDA and AMPA receptors are important mediators of neuronal LTP. The NMDA receptor is an ionotropic receptor for glutamate which forms a heterotetramer between two NR1 and two NR2 subunits. Ovariectomy was shown to cause a decrease in binding and expression of the NR1 and NR2B receptor subunits in the rat hippocampus (Cyr et al., 2001) and estradiol replacement caused an increase in their expression levels (Gazzaley et al., 1996). The increase in NMDA receptor binding and expression correlated with the increase in NMDA receptor-mediated synaptic input. In hippocampal slice cultures and in vivo, estradiol-induced increase in CA1 spine density is dependent on NMDA receptor synaptic input (Woolley and

McEwen, 1994, Woolley et al., 1997). Collectively, the findings suggest that E2 enhances NMDA receptor-mediated neurotransmission in hippocampal cells which may lead to its rapid effects on neuronal LTP. The present study also demonstrated an increase in the expression of NR1 subunit of NMDA receptor with E2 replacement which is quite consistent with the findings from other studies.

Accumulating evidence from many studies has shown that integrins are involved in multiple forms of synaptic plasticity and memory formation. Infusion of hippocampal slices with integrin-activating RGD-peptides caused an increase in the amplitude and duration of NMDA receptor-mediated synaptic currents. The treated hippocampal slices also showed an increase in the tyrosine phosphorylation and subsequent activation of NR2A and NR2B subunits of the NMDA receptor (Bernard-Trifilo et al., 2005). These effects were blocked when treated with function-blocking antibodies against β1-integrin or Src kinase inhibitor PP2 (protein phosphatase 2). A rapid increase in β1-integrin dependent protein tyrosine phosphorylation of focal adhesion kinase (FAK), proline-rich tyrosine kinase (PYK 2) and Src family kinases was observed when synaptoneurosomes isolated from adult rat forebrain were treated with RGD-peptides (Bernard-Trifilo et al., 2005). Moreover, RGD-peptides also enhance the slope and amplitude of the excitatory post-synaptic responses by modulating AMPA-type glutamate receptors and this effect was shown to be blocked by function-blocking antibodies against a variety of integrin subunits, NMDA receptor antagonist, CaMKII (Calcium-Calmodulin kinase II) inhibitor or Src tyrosine kinase inhibitor PP2 (Kramar et al., 2003). Collectively, these findings indicate that activation of β1-integrins can trigger the activation of the kinase signaling cascade Src/FAK/PYK2/CaMKII which then can enhance the NMDA and AMPA-receptor function. Therefore, the integrin-mediated signaling plays a major role in regulating neuronal LTP. Another hypothesis is that

integrins may regulate neuronal LTP and synaptic plasticity by interacting with actin cytoskeleton and reorganization of actin cytoskeleton.

Extensive studies on estrogens suggested that estrogens can regulate neuronal LTP through the enhancement of NMDA and AMPA receptor-mediated synaptic input via the activation of NMDA and AMPA receptor expression that may lead to the increase of dendritic spine density in the hippocampal CA1 region which may transform to the induction of LTP and memory formation. On the other hand, studies have also pointed out a major role for integrins in regulating neuronal LTP. It was shown that activation of integrins trigger integrin-related kinase signaling cascades which may then enhance NMDA and AMPA receptor-mediated synaptic input. Although, some of the links or the processes involved in the estrogen and/or integrin regulation of synaptic plasticity have been identified, there still are many questions that need to be addressed to get a better understanding of this complicated physiological process. One aspect that would be very interesting to understand is that how integrin activation is initiated for all the events involved in the integrin-mediated regulation of LTP or synaptic plasticity. Also, there are no reports published to date suggesting a direct association between estrogens and integrins with regards to their combined involvement in the events leading to synaptic plasticity. In the present in vivo study, though we expected to see an effect of E2 replacement on the expression of β1-integrins based on the results from the in vitro experiments, there were no significant differences in β1-integrin expression observed within E2 treated animals. Much research needs to be done to determine if there is any cross-talking between estrogen signaling pathways and integrin signaling pathways since both the signaling pathways have a major role in the processes of learning and memory. Given the importance surrounding β1-integrins in the events leading to

neuronal LTP, we cannot rule out the possibility of the role that β1-integrins might have in E2-mediated synaptogenesis and memory formation.

CHAPTER VI

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