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ABSTRACT

The enhancing effects of 17-beta estradiol (E2) on performing cognitive tasks has been well demonstrated in laboratory mice, rats, and primates. Also, there is ample clinical evidence indicating E2 enhances memory and reduces risk for Alzheimer's disease. Furthermore, by increasing the capacity for long-term potentiation (LTP) in the hippocampus, E2 effectively increases the synaptic plasticity of this brain region in a manner that correlates with memory formation. The molecular mechanisms underlying LTP and synaptic plasticity have largely focused on the role of E2-induced signal transduction in the nucleus, and regulation of plasticity related gene expression at the transcriptional level. Conversely, the idea that E2-induced signaling regulates at the level of translation and may play a role in these processes has yet to be explored. Recently, extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) signaling pathways have been shown to couple synaptic activation to protein synthesis machinery. Here we investigate translational control by E2-induced ERK and mTOR signaling in primary neuronal culture. E2-induced signaling resulted in enhanced

phosphorylation of ribosomal protein (S6) and eIF4E binding protein 1 (4EBP1) in an ERK and mTOR-dependent manner. Neuronal activity-dependent ERK and mTOR signaling have been shown to induce translation of a diverse array of dendritic resident mRNAs, including α-CaMKII and GluR1 subunits. Using a green fluorescent protein (GFP) translational reporter, we demonstrated that E2 stimulates GFP protein synthesis. We have also demonstrated that E2 treatment of hippocampal neurons increases surface expression of GluR1. Taken together, our results provide a mechanism by which E2 modulates the components necessary for persistent forms of LTP and long-term depression (LTD).

TRANSLATIONAL CONTROL BY ESTROGEN-INDUCED

SIGNALING IN PRIMARY RAT HIPPOCAMPAL NEURONS

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TRANSLATIONAL CONTROL BY ESTROGEN-INDUCED SIGNALING IN PRIMARY RAT HIPPOCAMPAL NEURONS

THESIS

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CHAPTER 1

INTRODUCTION

Estrogen Effects on Cognitive Function

Estrogens have direct effects on brain regions that control cognition. In animals (rodents and primates) most investigators have found evidence that the feminizing hormone 17β-estradiol (E2) enhances synaptic plasticity and improves the performance of hippocampal-dependent cognitive tasks. Varying E2 levels have even been shown to influence memory processing in humans. These elevated levels of E2 are positively correlated with enhanced short term memory. When E2 levels fall, there is a temporary decline in memory processing [1]. Cyclical changes in endogenous levels of E2 modulate the induction of long-term depression (LTD) and long-term potentiation (LTP) in the hippocampus [2]. Long lasting increases and decreases in synaptic strength are known as LTP and LTD, respectively. The mechanism(s) by which E2 modulates the induction of LTP and LTD are not known. The variation of performance during neuropsychological tasks in different sexes and phases of the menstrual cycle correlates with the cortical activation pattern as measured by functional Magnetic Resonance Imaging (fMRI) [3]. Low-dose E2 treatment in post-menopausal elderly women facilitates the efficiency of brain function during the performance of sustained attentional

tasks. This enhanced performance was correlated with the altered activity of the cortical and sub-cortical brain regions [4].

Estrogen's Role in Hippocampal Formation

Research by various investigators has uncovered cellular and molecular correlates of estrogen's modulation of hippocampal function. These include effects on cell morphology, synapse formation, membrane excitability, cell signaling pathways, neurotrophin systems, and neurogenesis.

Spines and Synapses

E2 increases spine density in rat primary hippocampal neurons [5]. The fluctuation of spine and synapse density occurs during the natural estrous cycle in rats. Rats in proestrous phase have 30% higher spine density in their CA1 region, than rats in late estrous phase [6]. The density of dendritic spines on hippocampal CA1 pyramidal neurons has been correlated with the formation of associated memories in both hippocampal and non-hippocampal tasks [7]. Recent evidence suggests that E2-mediated increases in spine synapse density are accompanied by increases in molecular components of the spine and synapse apparatus. In cultured hippocampal neurons, E2 increases synaptophysin, spinophilin, and post-synaptic density-95 (PSD-95) immunoreactivity [8], suggesting E2 also increases spine density *in vitro*. A few studies using non-human primates support the conclusion from rodent studies that E2 enhancement of hippocampal formation correlates with modulation of the dynamics of spine structure [9].

Excitability, Long-Term Potentiation (LTP), Long-Term Depression (LTD) and Memory

Estrogen's ability to increase excitability and synaptic potentiation may underlie the mechanism by which E2 enhances hippocampal-dependent learning and memory. E2 rapidly potentiates kainite-induced currents in hippocampal neurons from wild type, and ER-alpha knock-out mice [10]. Rapid spine synapse formation was observed with treatment of 17-alpha and 17-beta isomers in the CA1 hippocampal region of ovariectomized (OVX) female rats [11]. Rapid enhancement of visual and place memory by E2 has also been observed in rats [12]. Acute application of E2 in hippocampal slices increases N-Methyl D-Aspartate (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) transmission and induces LTP [13]. E2 facilitates cholinergic neurotransmission in the septal hippocampal pathways [14] and induces improvement in working memory performance [15]. It has also been shown that E2 rapidly modulates excitability in rat medial amygdale [16] and hippocampal neurons [17]. Modulation of E2 levels in pre-, peri-, and post-menopausal women affects neuronal activity as measured by fMRI in a variety of brain regions during the performance of cognitive, [1] and sustained attentional task [4]. Although underlying mechanisms by which E2 induces these effects remain largely unknown, activation of cell membrane-initiated signaling events that increase intracellular Calcium (Ca^{2+}), and cyclic adenosine monophosphate (cAMP) levels, and lead to activation of Src, G-protein coupled receptor (GPCR), mitogen-activated protein kinase ERK, protein kinase B (Akt), protein kinase A (PKA), phosphatidylinositol 3 kinase (PI3K), and adenylyl cyclase [18] appear to be important. We do not yet understand how E2-induced changes in the

neuroanatomy and neurophysiology of developing and adult organisms alter cognitive function; however, it has become increasingly clear that E2 plays an important role in learning and memory.

NMDAR-Mediated Neurotransmission by E2

Various studies have implicated NMDAR-mediated transmission in estrogen's ability to regulate hippocampal spine density. Ovariectomy causes a decrease in NMDAR binding and expression of NR1 and NR2B subunits in the rat hippocampus, and E2 replacement increases their expression [19]. Since NMDARs are the major facilitators of neuronal LTP, the ability of E2 to increase NMDAR-mediated input may account for its rapid effects on neuronal excitability and LTP [20].

Effects of E2 on the Inhibitory Neurotransmission of GABA

In vitro experiments indicate that E2-induced decreases in GABAergic inhibition result in the increase in spine density of CA1 pyramidal cells [21]. However, ovariectomy decreases and E2 increases glutamic acid decarboxylase (GAD) protein expression in the CA1 pyramidal cell layer [22]. In hippocampal slices E2 treatment transiently decreased GABAergic inhibition, followed by a recovery of the inhibition and an increase in GAD expression [23]. This recovery may indicate that E2-mediated excitation of pyramidal cells might lead to excitotoxicity and even seizures. Thus, effects of E2 could serve, on one hand, to facilitate increased NMDAR input, and on the other, to maintain excitation.

Signaling Pathways

Not only does E2 rapidly affect neuronal excitability, but it also activates cell signaling pathways in neurons. The PI3-K/Akt pathway has been implicated in E2 effects on synaptogenesis and increased spine density [8]. AKT activation may promote translation of new spines and synaptic proteins upon E2 stimulation. The AKT pathway plays a well known role in cell survival, and has been implicated in E2s neuroprotective actions [24]. E2 causes phosphorylation of cAMP-response-element-binding protein (CREB) in both cultured neurons and *in vivo* [25]. CREB phosphorylation has been implicated in E2-induced spine formation, and upregulation of synaptic protein synthesis in hippocampal neurons [26]. *In vivo*, E2 activation of CREB occurs in the CA1 and CA3 regions of the hippocampus. E2 also activates CREB in neurons from the basal forebrain and cortex [27, 28]. Several recent studies demonstrate E2 activation of ERK/CREB in hippocampal neurons is mediated by rapid calcium influx seen within minutes of E2 exposure [29]. E2 activation of extracellular regulated-signaling kinase (ERK) and CREB may be crucial for its downstream enhancement of hippocampal dependent learning and memory. Although this hypothesis has yet to be proven, ERK plays a crucial role in synaptic plasticity and memory [30]. α-CaMKII also plays an important role in synaptic plasticity, learning, and memory [31]. Recent studies have implicated α-CaMKII in E2's rapid effects in the hippocampus. In primary hippocampal neurons, E2 activation of CREB depends not only on ERK but also on α-CaMKII. *In vivo*, α-CaMKII is activated in mouse hippocampal formation within an hour of peripheral E2 injection [32].

Summary

Several of E2's effects discussed above in the hippocampal formation may account for the hormone's enhancing effects on learning and memory. E2 rapidly activates signaling pathways, leading to synaptic protein translation and modification, actin remodeling, and dendritic spine and synapse formation. In addition, E2 rapidly induces calcium currents through L-type calcium channels, increases NMDAR-mediated excitation, and decreases GABAR-mediated inhibition on hippocampal pyramidal cells. E2 also influences the expression of multiple ligands and their receptors in the hippocampus, including neurotrophins such as BDNF. E2 may directly activate signaling pathways, neurotrophin and neurotransmitter systems in the basal forebrain, which projects cholinergic neurons to the hippocampus and indirectly impact hippocampal function.

LTP, LTD, and Protein Translation

Long-lasting changes in synaptic strength require networks of biochemical cascades in pre- and post-synaptic neurons. Biochemically, both LTP and LTD inducing stimuli enhance ERK [33, 34] and mammalian target of rapamycin (mTOR) dependent translation of diverse sets of mRNAs [35-38]. ERK activation is required for translational induction in response to neuronal activity, LTP, and hippocampal memory formation, through regulation of translation initiation factors [34]. Hippocampal dendrites contain mRNAs for α-CaMKII, NMDAR1 (subunit 1), activity-regulated cytoskeletal protein (*arc*), and the AMPAR (GluR1 subunit) [39-41]. Furthermore, all of the components necessary for protein synthesis are present in dendrites. It has been

shown that after LTP induction, polyribosomes, as well as, α-CaMKII are recruited into spines [42, 43]. All of these studies suggest local protein synthesis is required for synaptic plasticity.

Neurons use at least three different strategies for activity-dependent regulation of protein synthesis and targeting. First, some proteins are synthesized in the soma where they are translated from newly transcribed mRNAs. For example, transcription factors such as CCATT enhancer binding protein (C/EBP) [44] and CREB [45] are both activated in response to certain forms of synaptic stimulation. These remotely synthesized proteins are subsequently delivered to the appropriate synapse via the generation of "tags" at activated synapses [46]. Secondly, newly transcribed mRNAs are transported to activated synapses where they are translated. This mechanism is true for *arc,* an immediate-early gene whose transcription is regulated by synaptic activity [47]. A third strategy that neurons use to achieve activity-dependent regulation of protein synthesis and targeting is by regulating translation of mRNAs localized at synapses. Localized synthesis was suggested based on the observation that dendritic shafts/spines contain polyribosomes, tRNAs, translation initiation factors, and specific mRNAs [48-50]. Several mRNAs are known to be localized *in situ.* The mRNAs include, but are not limited to, those encoding microtubule-associated protein 2 (MAP2), *arc*, fragile X mental retardation protein (FMRP), α-CaMKII, NMDAR1, and AMPAR [51].

It is generally thought that proteins required for neuronal function are made in the cell body (with the exception of the mitochondria). However, in 1982 it was discovered that polyribosomes are located near the base of many dendritic spines in the hippocampus[48]. This suggested the possibility that neuronal proteins can be locally

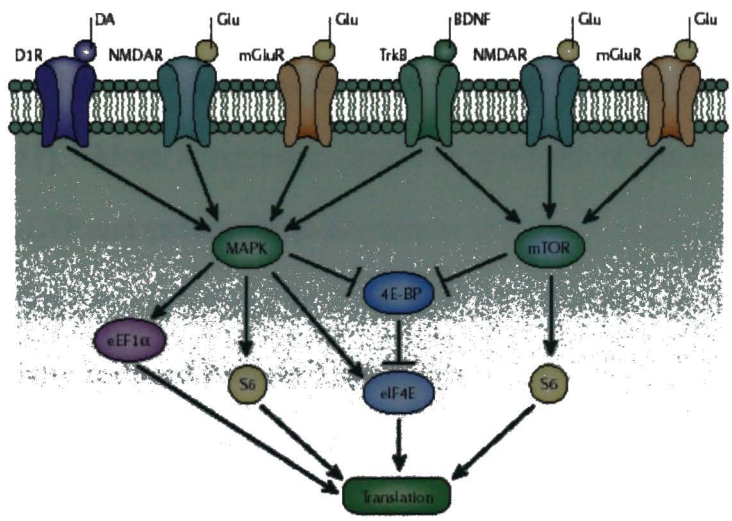
synthesized in dendrites. This local, rather than, somatic synthesis of proteins at synaptic sites, suggests certain cell-biological mechanisms are in place that allow synapses to independently control their own strength, bypassing the need for protein trafficking from the soma [41]. The ability to locally synthesize proteins in the dendrites helps synapses solve the problem of maintaining specificity, while obtaining newly synthesized proteins that are necessary for maintaining synaptic plasticity [52-54]. In recent years several studies have shown that locally synthesized proteins likely contribute to long lasting synaptic plasticity [55].

The results of a recent Women's Health Initiative Memory Study (WHIMS) indicated a deleterious effect of combined conjugated equine E2 with respect to a risk for Alzheimer's or cardiovascular disease [56]. Multiple factors may be potential contributors to discrepant inferences from observational research, WHIMS, or trial of Hormone Therapy (HT) in women with existing Alzheimer's disease. Prime among these factors are age, dose and composition of hormones, and timing of hormone therapy initiation in relation to menopausal stage. These factors may be responsible for treatment outcomes on dementia risk. Future research will help us better understand and isolate the mechanism of E2's beneficial effects in cognitive function so that a more correct judgment can be made regarding the risk and benefits to patients undergoing estrogen therapy (ET).

Synaptic Plasticity and Protein Translation

The neuron functions by modifying its response to synaptic input in an experience-dependent way. Long lasting increases and decreases in synaptic strength are

known as LTP and LTD, respectively. In synaptic plasticity, induction of the early phase of LTP (E-LTP) and the early phase of LTD (E-LTD) does not require enhanced translation. However, this process is essential for induction of the late phase of LTP (L-LTP) and the late phase of LTD (L-LTD), which are known to be synaptic correlates of long-term memory (LTM). Although L-LTP and L-LTD are manifestations of opposite synaptic strength changes, the induction of both requires upregulated translation. LTP induced by high frequency stimulation or brain-derived neurotrophic factor (BDNF), and metabotrophic glutamate receptor (mGluR) dependent LTD use signaling pathways to regulate translation initiation in the hippocampus as shown in the illustration below.



Regulation of neuronal activity-induced translation via MAPK and mTOR signaling pathways. [57]

The above schematic shows the molecular mechanisms responsible for translational enhancement. Neuromodulators such as dopamine, acetylcholine, noradrenaline, glutamate (via both NMDAR and mGluR), and BDNF activate the ERK/MAPK pathway. Glutamate and neurotrophins activate the mTOR pathway. In turn, these pathways activate translation of most mRNAs present in dendrites, by promoting the phosphorylation of eukaryotic translation initiation factor 4E (eIF4E), small ribosomal protein (S6), eukaryotic elongation factor 1α (eEF1α), and via, phosphorylation of eIF4E binding protein (4EBP) which negatively regulates eIF4E [57].

Dendrites can translate mRNA, and new protein synthesis is induced in dendrites after exposure of cultured neurons to BDNF, and glutamate receptor agonists [58-60]. A number of mRNAs that encode proteins involved in synaptic plasticity, including α-CaMKII, microtubule-associated protein 2 (MAP2), AMPAR and *arc*, have been found in dendrites [51]. More importantly, dendritic synthesis of the α-CaMKII protein is essential for L-LTP and various forms of memory [43].

Neuronal Activity Induced Protein Translation

Translation of eukaryotic mRNAs is a multi-step process that is primarily regulated at the level of initiation. Experiments in mitotic cells have demonstrated 5' cap recognition and ribosomal recruitment by translation initiation factors as key events in this multi-step process [61]. Translation of the 5' cap accounts for the synthesis of the vast majority of cellular proteins, since all mRNAs transcribed in the nucleus have a 5'cap. Recognition of the cap is accomplished by eIF4E and eIF4G. After recognition, the eIF4E-associated factor, eIF4G, then recruits the 40S ribosomal subunit. Ribosomal

protein S6 also plays a major role in this coordinated control of protein synthesis and cellular growth [61].

Dendritic Protein Synthesis

The functional significance of dendrititc protein synthesis was discovered by demonstrating a requirement for local protein synthesis in BDNF induced synaptic plasticity [62]. It is now clear that dendritic protein synthesis is required for many forms of long-term synaptic plasticity, raising the possibility that similar local translational control contributes to various aspects of memory processing. Recent studies have suggested that translation in dendrites related to neuronal plasticity is critical, and somatic translation may be dispensable. For example, BDNF induces potentiation of CA3-CA1 synaptic transmission in hippocampal slices where CA1 dendrites have been surgically dissected out from the soma and the induced potentiation still requires protein synthesis [62]. Recent studies show activation of mGluRs or paired-pulse low frequency stimulation in hippocampal slices can induce LTD that requires dendritic, but not somatic, protein synthesis [63]. Also, hippocampal slices prepared from transgenic mice lacking the 3' UTR dendritic targeting element of the α-CAMKII mRNA, exhibited severe reduction of LTP [64]. Several recent studies have also demonstrated that isolated hippocampal dendritic fields can support dendritic protein synthesis-dependent form of LTP [35, 65, 66]. In another study, it was demonstrated local protein synthesis is required for a rapid form of synaptic plasticity mediated by a D1/D5 receptor agonists [40]. This rapid form of synaptic plasticity is mediated by an increase in the number and size of synaptic GluR1 proteins.

The regulation of local translation is also important for ongoing levels of synaptic acitivity present in neural networks. The inhibition of spontaneous neurotransmission in cultured hippocampal neurons resulted in rapid enhancement of dendritic protein synthesis, indicating spontaneous neurotransmission inhibits local translation [67].

Localization of the Translation Machinery

A number of studies have demonstrated that the components necessary for translation are constitutively present in dendrites [36, 49, 68, 69]. Moreover, some of these components have been shown to exhibit activity-dependent trafficking within dendrites [42, 58, 70]. In addition, the presence of endoplasmic reticulum and golgi elements in dendrites, and the capacity for post-translational modification and membrane insertion of newly synthesized proteins have been reported in the literature [71].

Studies using *in situ* hybridization have identified more than thirty different mRNA species in dendrites [72]. These mRNAs are transported to dendrites and docked at the appropriate synaptic sites. The mechanisms responsible for transporting mRNAs to dendrites are thought to involve recognition of cis-acting elements in the 3' UTR, by specific RNA binding proteins that interact with microtubule based transport systems [73]. The importance of mRNA trafficking to dendrites with respect to synaptic function has been demonstrated by a recent study that examined mutant mice in which the native 3'UTR of α-CaMKII mRNA was replaced with 3'UTR of bovine growth hormone [64]. Dendritic localization of α-CaMKII mRNA was completely abolished in these mice, and the protein levels were also severely reduced. Trafficking of mRNA to dendrites is not just a constitutive process; it can also be regulated by synaptic activity [47, 74]. This has

been demonstrated in studies of the regulated delivery of mRNA encoding *arc* to synaptic sites after high frequency stimulation [47]. Thus, mRNA localization can be tightly controlled within dendrites in an activity-dependent manner, and this process seems likely to be a contributing factor to input-specific synaptic plasticity.

Signaling to the Translation Machinery

Two signal transduction pathways, ERK and PI3-K, have emerged as key regulators of translational efficiency in multiple types of eukaryotic cells. Both pathways are activated downstream of various growth factors, and act as intermediates between the transduction of environmental signal and the control of both translation and transcription. In addition, both pathways have been shown to play important roles in synaptic plasticity and memory [75] [76]. Another signaling molecule in translational regulation is mTOR. mTOR activity is the target of protein synthesis inhibitor rapamycin, which inhibits translation of mRNAs [61].

Mechanism for Translation and its Regulation

The process of protein synthesis is comprised of three phases: initiation, elongation, and termination. Each of these phases is subject to regulation.

Initiation

The 5' cap structure (7-methyl GTP cap) that acts in translation initiation is recognized by the eIF4E protein complex comprised of the cap binding protein eIF4E, the RNA helicase eIF4A, and the scaffold protein eIf4G. eIF4G then recruits the 43S pre-initiation complex (composed of 40S ribosomal subunit and GTP-bound eIF2 complexed

with the initiator Met-tRNA) to the mRNAs 5'end through interaction with eIF3. The mRNA-bound ribosomal complex then scans down the mRNA until an initation codon is reached. The protein factors then dissociate from the complex and the 60S ribosomal subunit joins. The newly formed 80S ribosome with the met-tRNA in the ribosomal P-site initiates on the mRNA and begins elongation.

The cap binding protein eIF4E is a rate limiting factor for translation initiation, and its regulation by phosphorylation is an important mechanism for controlling protein synthesis. The direct phosphorylation of eIF4E is controlled by the MAPK/ERK signaling pathway. In most cases, overall translation rates are correlated with the extent of eIF4E phosphorylation [77]. The 40S ribosomal protein, S6, is also a target of phosphorylation and its enhanced phosphorylation correlates with induced translation initiation. Specifically, phosphorylated S6 enhances initiation of those mRNAs that contain a 5' terminal oligopyrimidine tract (known as 5' TOP mRNA family). In general, the majority of the TOP family of mRNAs consists of members that encode components of the protein synthesis machinery; thus S6 phosphorylation could provide a potential mechanism for regulating translation in dendrites. Cytoplasmic phosphorylation of S6 is mediated by P70S6 kinase (S6K), which in turn is regulated by mTOR, as well as, by downstream effectors of the PI3-K pathway [78, 79].

Elongation

During the elongation phase amino acids are added to the growing polypeptide as the ribosome translocates one codon at a time relative to the mRNA. At present there are three elongation factors that have been identified in mammalian cells. Two of these

factors, eukaryotic elongation factors 1α and 1β (eEF1α and eEF1β), are responsible for recruiting a new amino acyl tRNA to the ribosomal A site. Phosphorylation of eEF1α/1β by calcium-dependent classical PKC isoforms, enhances their activity in translation elongation. The third elongation factor, eEF2, is necessary for the translocation of ribosomes along the mRNA. Activity of eEF2 is controlled by phosphorylation. eEF2 phosphorylation in neurons has been shown to be regulated in an activity-dependent manner [59, 80].

Termination

Translation termination requires two release factors, eukaryotic release factor 1 (eRF1) which recognizes all three stop codons and catalyzes the termination reaction, and eukaryotic release actor 2 (eRF2) which stimulates eIF1 in a GTP and ribosome dependent manner [81]. While regulation of termination has yet to be established in the neuronal system, dissociation of mRNA from ribosomes and recycling of ribosomes into initiation mode, should play an important role in dendritic translation.

Cytoplasmic Polyadenylation

Translation of a specific class of mRNAs can also be induced by cytoplasmic polyadenylation of the 3' tail. This class of mRNAs contains short nucleotide sequences in the 3'UTR known as cytoplasmic polyadenylation elements (CPEs). These sequences are recognized by the CPE binding protein (CPEB), which represses translation via

interaction with the protein maskin. Maskin then binds eIF4E and inhibits its association with eIF4G [82]. Phosphorylation of CPEB by the protein kinase aurora can relieve this repression. One dendritically localized mRNA that can be regulated in this way is α-CaMKII [83]. α-CaMKII mRNA contains two CPEs, and these are required for α-CaMKII translation induced by either visual input or NMDAR activation [83, 84]. NMDAR activation in synaptosomes has been shown to stimulate aurora activity, phosphorylation of CPEB, and induce α-CaMKII mRNA translation [85]. Recently it has been demonstrated that α-CaMKII can phosphorylate CPEB at synapses, leading to enhanced CPEB-induced translation [86].

Neuromodulatory Regulation of Dendritic Translation

BDNF is known to promote structural changes in the CNS. Studies of BDNF knockout mice suggested a specific role of this growth factor in protein synthesis dependent synaptic plasticity [87, 88]. The application of BDNF to hippocampal slices induces a form of LTP that is dependent on protein synthesis [62]. BDNF-induced LTP occurs in dendrites that have been surgically isolated from their parent cell bodies, but is blocked by protein synthesis inhibitors.

Studies of the effect of BDNF on translational responses in neurons, indicates that it is a strong activator of both overall translation in the cell body and local translation in dendrites [34, 89, 90]. The ability of BDNF to induce activation of translation in neurons is regulated by a number of proteins important for translation initiation [91]. In both cortical and hippocampal neurons, BDNF induced the phosphorylation of eIF4E and eIF4E binding protein 1 (4EBP1) through an ERK-dependent mechanism [34]. BDNF

also stimulated the phosphorylation of ribosomal protein S6 in hippocampal neurons in an ERK-dependent manner [34].

Significance of Project/Hypothesis

During the estrous cycle, ribosomes are recruited in the dendrites of hippocampal neurons. Furthermore, E2 activates ERK and Akt, the kinase involved in the mTOR signaling pathways. If increased amounts of E2 in the blood during the estrous cycle cause polyribosomes to accumulate in dendrites of the hippocampus, then E2 should induce protein translation in dendrites of hippocampal neurons via phosphorylation of ERK and/or mTOR kinase. The activation of these kinases should induce phosphorylation of mRNA translation factors that in turn, lead to translation of dendritic resident mRNAs.

The purpose of this study was to determine: 1) whether E2 can induce activation of translation initiation factors via ERK and/or mTOR kinases, and 2) whether E2-induced activation of translation initiation factors are responsible for mechanistically linking dendritic mRNA translation.

These studies may provide new insights into the mechanism(s) by which E2 and its non-feminizing derivatives control short- and long-lasting forms of synaptic plasticity and memory. These studies have clinical implications for the therapeutic use of non-feminizing E2 in preventing memory loss without the harmful effects of combined conjugated equine E2 such as potentially increased risks for Alzheimer's disease or cardiovascular diseases.

CHAPTER 2

MATERIALS AND METHODS

Culturing Hippocampal and Cortical Neurons from Fetal Rat Brain

Removal of Embryos

In a laminar flow hood, the abdomen of a pregnant Sprague-Dawley rat (embryonic day 18-Charles Rivers, USA) was sterilized with excess 70% ethanol. Using large forceps, the skin of the abdomen was lifted approximately 1 inch proximal to the gonads. An incision on the midline through the skin was made starting from the pelvis continuing to the thorax, carefully avoiding incision of the muscles underneath the skin at this time. The abdominal wall was lifted at the point of original skin incision, and then an incision through the abdominal wall was made to reveal the uterus (dark, purple-colored tissue) containing the embryos. The embryos were lifted in tandem out of the uterus. Some cutting with small scissors was needed along the placenta to free the embryos from the uterus. The embryos still in tandem were placed in a large culture dish. A small incision above the placenta allowed each individual embryo to be freed from the embryonic sac. The embryos were subsequently decapitated and the heads transferred to a new dish containing Dulbecco's Modified Eagle's Medium (DMEM).

Removal of Brains

Dumont forceps were used to stabilize the head on the bottom of the dish. The tips of curved Dumont forceps were inserted into the forehead at the level of the eyes to slightly lift the head from the dish. With small scissors, a cut was made along the middle vein through the skin and the skull until reaching the forceps in the forehead. Using the dull edge of the scissors to slide over the skull in a rostral to caudal direction, the brain was released from the skull through the caudal incision. The brains with intact hemispheres and brain stems were placed into a dish with fresh DMEM.

Removal of Hippocampi

Hippocampi were removed under a dissecting microscope. A combination of scissors and Dumont forceps were used to cut and gently separate the hemispheres from the diencephalon and the brain stem. In most cases, the hemispheres were obtained without thalamus tissue. The meninges were removed from the separated hemispheres. The hemispheres were positioned on their lateral side with the medial side facing up. In this position the hippocampi were clearly visible and gently pinched away avoiding cortical tissue. After dissection, the hippocampi were transferred to a conical tube containing DMEM.

Preparation of Hippocampal Cell Suspension

After all hippocampi had been dissected, the tissue suspension was dissociated using a Pasteur pipette. Once the solution became cloudy, the tissue was further

dissociated using fine-polished Pasteur pipettes, the tips of which were reduced to one-half and one-third respectively, of the normal size. After triturating the tissue, the cell suspension was filtered through an 80 micron filter to obtain single cell suspension. This resulting suspension was plated on poly-L-lysine coated cover slips or petri dishes containing neurobasal medium supplemented with B27, and cultured *in vitro* in 95% humidity and 5% CO_2 atmosphere. On day 2, cells were treated with 5μM arabinosylcytosine hydrochloride (AraC) to inhibit glial cell growth. At 7-8 days growth, microtubule-associated protein 2 (MAP2) found exclusively in nerve cells and glial fibrilary acidic protein (GFAP) immunofluorescence staining and confocal microscopy confirmed 99% of the cell population were neurons.

Transfection of Primary Neuronal Culture

Primary hippocampal neurons (7-10 days *in vitro* (DIV) were transfected with pcDNA3.1-5′ dGFP3′ using lipofectamine reagent (Invitrogen Carlsbad, CA, USA), according to the manufacturer's protocol. The plasmid, (pcDNA3.1- 5′ dGFP3′ construct) used in our study as a fluorescent translation reporter gene, was a kind gift from Dr. Erine Schuman [92].

Time Lapse Imaging of Dendrites Expressing Fluorescent Translation Reporter

Approximately 24-48 hours after transfection, neurons on cover slips were placed in a 35 mm dish containing the complete culture medium. Healthy pyramidal neurons expressing GFP were chosen for experimentation. E2 (10 nM) was added to the medium and images were acquired at 20 minute intervals using laser-scanning microscopy (LSM).

Immunoblotting

Cells were lysed with cold radio immunoprecipitaion assay (RIPA) buffer containing protease and phosphatase inhibitors (1% NP40 containing 150 mM NaCl, 2 mM EDTA, 1% deoxycholate, 200 μg/mL aprotinin, 100 μg/mL pepstatin, 50 μg/mL leupeptin and 10 mM benzamidine, 20 mM NaF, 10 mM Na-ortho vanadate). Before electrophoresis, protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples (30 μg total protein) were resolved using SDS-polyacrylamide gel electrophoresis, and transferred into Immobilon-P membrane. Rabbit polyclonal antisera against dually phosphorylated ERK1/2 (Thr202/Tyr204), phospho-S6 (S235/S236), and phospho-4EBP1 (S65) (Cell Signaling, USA) were used for detection. Blots were stripped and reprobed with antisera directed against total ERK1/2, S6, and 4EBP1 (Cell Signaling, USA). Results were quantified using Image J, (National Institute of Health), calculated as the ratio of phosphorylated species to total, and then normalized to the untreated control condition.

Immunofluorescence Procedures for Laser–Scanning Confocal Microscopy

Imaging studies conducted using live hippocampal neurons grown on poly-L-lysine-coated cover slips, were incubated with rabbit anti-GluR1 N-terminal domain antibody for surface labeling, (EMD Biosciences, Gibbstown, NJ) at room temperature for 40 minutes and then washed twice with HEPES buffer saline (HBS). Immunostaining was visualized by incubating Alexa 633 conjugated (Invitrogen-Molecular Probes, Carlsbad, CA) anti-Rabbit secondary antibody for another 30 minutes at room

temperature, followed by three 10 minute washings with HBS. Cover slips were mounted with anti-fade (Invitrogen-Molecular Probes, Carlsbad, CA). A laser-scanning confocal imaging system (LSM 510, Zeiss) was used for image acquisition and processing. Alexa 633 was excited with the 633 nm line of He-Ne ion laser and emitted light was collected at approximately 665 nm. To compare between cover slips, all images were acquired at the same settings without knowledge of the experimental conditions during image acquisition. All post-acquisition processing and analyses were conducted with Image J and Mattlav (The Math Works, Inc.) Quantification of fluorescent signal in dendrites (as a function of distance) was done by linearizing the dendrites first and then extracting from the image using a modified version of the straighten plugin for Image J.

CHAPTER 3

RESULTS

Estrogen Induces ERK and mTOR Dependent Phosphorylation of Translation Initiation Factors

Estrogenic steroids exert multiple effects on CNS neurons. E2 enhances synaptic transmission through both NMDARs and AMPARs. E2 modulates LTP and LTD. E2 activates kinases such as ERK, and AkT. E2 increases dendritic spine size and synapse density. These observations, along with others, indicate E2 potentially acts as a modulator of synaptic plasticity. Modulation of synaptic activity requires new protein synthesis. Recent work has implicated local dendritic protein synthesis in LTP and LTD. LTP and LTD inducing stimuli enhance translation of a diverse set of dendritic resident mRNAs such as *arc*, α-CaMKII, AMPAR (GluR1, and GluR2 subunits), and eukaryotic translation initiation factor 1A (eIF1A). Mechanistically, the increase in mRNA translation is characterized by ERK and/or mTOR-dependent increases in phosphorylation of eukaryotic translation initiation and elongation factors. We hypothesized that estrogens and their non-feminizing derivatives enhance ERK and/or mTOR-dependent phosphorylation of eukaryotic translation initiation factors, thereby increasesing translation of dendritic resident mRNAs. Using western blot analysis and phospo-specific antibody we tested this hypothesis by measuring the phosphorylation

status of ERK, and the translation initiation factors, 4EBP1 and ribosomal protein S6, in E2 treated hippocampal neurons. As demonstrated in Figure 1A E2 (10 nm) induces phosphorylation of ERK 1/2 and ribosomal protein S6 within 10 minutes of treatment. Maximum phosphorylation of both ERK and S6 was observed at 30 minutes, and at 60 minutes phosphorylation decreased to near basal levels. Total ERK and S6 protein levels remained unchanged. Figure 1A also demonstrates that both ERK 1/2 and S6 phosphorylation were inhibited by a mitogen-activated protein kinase (MEK) inhibitor (U0126). E2 induced induction of phosphorylation of S6 and ERK and suppression of their phosporylation by U0126 was statistically significant as shown in figure 1B and 1C. Next we measured the phosphorylation status of 4EBP1. Hyperphosphorylation of 4EBP1 causes eIF4E release, enabling cap recognition and ribosomal recruitment. The hyperphosphorylation of 4EBP1 has been shown to be regulated by ERK and mTOR. Figure 1A also illustrates that E2 treatment rapidly induces phosphorylation of 4EBP1 and this induction is inhibited by U0126. In addition, we studied whether non-feminizing derivatives of E2 such as ZYC 3, ZYC 5, ZYC 26 also induce phosphorylation of S6 and if so, whether this induction is ERK and mTOR dependent. Non-feminizing derivatives of E2 have very low affinity binding with E2 receptor. The western blot in Figure 2 demonstrates that like E2, its analogues also induce phosphorylation of S6, and this induced phosphorylation is significantly reduced by inhibitors of ERK, PI3K and mTOR. Taken together, our results show that E2–induced ERK and mTOR signaling pathway modulate the phosphorylation state of translation initiation factors and this effect of E2 is estrogen receptor independent.

E2 Induces Dendritic mRNA Translation

Our observation of E2 induced S6 phosphorylation and the recent report that an E2 surge during the estrous cycle causes polyribosomes to accumulate in the dendrites of the hippocampus potentially indicate that E2 may induce dendritic mRNA translation. The mRNA for α-CaMKII is one of the most prominent dendritic mRNAs identified to date, and rapid synthesis of α-CaMKII has been observed during synaptic plasticity. We examined dendritic protein synthesis in living neurons by using a green fluorescent protein (GFP) reporter flanked by 5′ and 3′ untranslated regions (UTR) from the α-CaMKII mRNA. UTR of α-CaMKII mRNA contain information sufficient for its dendritic localization. GFP translation reporter 5′ GFP 3′ was introduced into rat primary hippocampal neurons using lipofectamine. Twenty-four hours after transfection, time-lapse imaging of live neurons was acquired from the initiation of E2 (10 nM) treatment for a period of 120 minutes, in 20 minute intervals. As shown in Figure 3 A-G, E2 induces spatiotemporal increments of GFP synthesis in the soma, as well as, in the dendrites of hippocampal neurons. Figure 4 shows the same two neurons visualized in figure 3 A-G with their dendrites labeled A-E. Figure 5A shows a time lapse collage of a dendrite taken from figure 4 labeled as A and straightened by image J. It is evident from the straightened dendrite that GFP fluorescence intensity increases in the distal area as time lapses. In the 3-D plot of fluorescence from the collage in figure 5B relative fluorescence intensity (reporter expression) is indicated by the color and the height of the pixels. A spatiotemporal increase in dendritic reporter expression is evident after E2 exposure.

E2 Increases Synthesis of AMPARs in the Dendrites of Hippocampal Neurons

Modulation of the levels of synaptic AMPARs by subunit trafficking, insertion, and internalization is a critically important mechanism for regulating LTP, LTD, and homeostatic synaptic plasticity. In one mouse model of Alzheimer's disease and fragile X syndrome, downscaling of AMPAR function is associated with substantial impairment of mechanisms involved in neural plasticity [93, 94]. An enriched environment largely rescued behavioral and neuronal abnormalities while increasing GluR1 levels in fragile X syndrome. Neuronal activity induces GluR1 mRNA translation in dendrites of neurons. Work from several laboratories has established that LTP inducing stimuli promote the physical insertion of AMPARs at the post synaptic membrane leading to an increase in AMPAR-mediated transmission of excitatory synapses. Because of E2's profound effects in synaptic plasticity, we hypothesized that like α-CaMKII, E2 also induces translation of GluR1 and subsequent insertion in the dendrites of rat hippocampal neurons. We tested the hypothesis by studying the effects of E2 on AMPAR synthesis in the dendrites of hippocampal neurons. E2-treated and untreated live hippocampal neurons were incubated with anti-GluR1 (N-terminal domain) antibody, which recognizes the extracelluar domain of GluR1, followed by Alexa 633 conjugated secondary antibody. Laser scanning confocal microscopy was used for image acquisition and processing. Relative to control, 30 minute E2 (10 nM) treatment led to an increase in surface expression as evidenced by increased intensity of red fluorescence (Figure 6). This E2-induced increase in GluR1 synthesis was inhibited by anisomycin (10 μg/ml), a protein translation inhibitor (Figure 6C). Quantitative analysis of E2-induced GluR1 synthesis in hippocampal dendritres (Figure 7) indicated the E2-induced increase in

synthesis and insertion of GluR1 in the membrane of dendrites was statistically significant. Fluorescence intensity data were subjected to ANOVA and the multiple range test Newman-Keuls, determined the pairwise comparisons of the means. Statistical significance was indicated with at least a $p < 0.05$.

CHAPTER 4

DISCUSSION

Protein synthesis is required for persistent forms of synaptic plasticity and memory formation. A key regulator of LTP-related protein synthesis is mTOR, which is beleived to modulate the translational capacity by facilitating the synthesis of particular components of the protein synthesis machinery. Recently, *arc* was shown to mediate plasticity related translation, an effect that may involve regulation of mTOR pathway [34].

It has been shown that estrogenic steroids exert multiple effects on central nervous system neurons, including potentiation of LTP and LTD. In both animals and humans, experimental results showed estrogens improve cognitive function. E2-induced modulation of synaptic plasticity and memory formation require new protein synthesis but little is known about the underlying regulatory mechanisms. We investigated the role of E2-induced ERK/Akt/mTOR signaling in these processes. In our current studies, we demonstrated E2-induced ERK activation was required for inducible phosphorylation of multiple factors that play a central role in the process of translation initiation. Specific phosphorylation of ribosomal protein S6 and 4EBP1 has been linked mechanistically to increases in translational efficiency in response to neuronal activity. Our observation is that these phosphorylation events are regulated coordinately with E2-induced signaling

mechanism. E2 treatment in hippocampal neurons leads to activation of ERK and Akt. The PI3K/Akt pathway has been implicated in E2 effects on synaptogenesis, spine number, and morphology. In hippocampus immunoreactivity for phosphor-Akt is increased in proestrous and estradiol-replaced rats relative to ovariectomized, estrous and diestrous rats [95]. In both midbrain and cortical neurons, E2 rapidly activates Akt suggesting that there is a direct action of E2 on these brain regions [27] [96]. In the mTOR pathway regulation of PI3K-generated phosphoionositides, colocalize PDK1 and its substrate Akt at the cell membrane. Phosphorylated Akt, in turn, indirectly activates mTOR by phosphorylation and inhibition of the mTOR suppressor TSC2 [97]. The mechanism by which ERK regulates mTOR is unknown, but studies in other systems have identified two proteins in the PI3K-mTOR pathway. ERK can directly phosphorylate PDK1 that is mediated by the ERK substrate RSK. At a more downstream site ERK can phosphorylate TSC2 and thereby inactivate TSC2 [98]. In neurons, the ERK signaling pathway is activated by stimuli associated with synaptic activity and plasticity. An important role for the ERK pathway in translational regulation was recently established in neurons. Our results indicate E2-induced ERK activation induces phosphorylation of specific residues in the ribosomal protein S6 (Serine 235/236) and the inhibitor of cap binding factors eIF4E and 4EBP1 (Serine 65). This E2-induced phosphorylation was significantly inhibited by U0126 (MEK inhibitor), rapamycin (mTOR inhibitor) and LY29402 (PI3K inhibitor). Thus, E2-induced activation of PI3K-Akt-mTOR signaling induced phosphorylation of ribosomal protein S6 and 4EBP1. E2-induced phosphorylation of translation initiation factors S6 and 4EBP1 maybe mechanistically linked to increased translation efficiency in activated neurons.

ERK and mTOR-dependent synthesis of translational machinery is observed in many cell types as an early response to stimuli that induce cell growth and this process depends on transcriptional and translational control over the expression of very specific classes of mRNAs [79]. As the enduring forms of synaptic plasticity and memory require new protein synthesis, the inducible translation of mRNAs in dendrites has been proposed as a control point in neuronal plasticity. It has been demonstrated that various mRNAs that encode proteins involved in synaptic plasticity, including α-CAMKII, MAP2, *arc*/ARG3.1, and the GluR1 subunits of AMPA receptors are locally translated after neuronal activation [63]. Importantly, dendritic synthesis of α-CAMKII protein is essential for LTP and various forms of memory [64]. We used the α-CAMKII GFP reporter [92] to investigate the role of E2 in induction of protein synthesis in dendrites of cultured neurons. Our results provide evidence that protein synthesis, in response to E2 treatment, accounts for the observed increased GFP fluorescence in dendrites of primary neurons. Anisomycin, a specific translation inhibitor, inhibited E2 induced GFP synthesis suggesting E2 induces local protein synthesis rather than other cellular processes such as mRNA and protein transport from cell bodies.

There is general agreement that stable incorporation of AMPARs into synapses occurs during LTP. Also it has been demonstrated that the GluR1 subunits of AMPARs are locally translated after neuronal activation. Our results demonstrate that E2 treatment in hippocampal neurons induces the synthesis in the membranes of hippocampal dendrites. The results reported here suggest E2 plays a potential role in inducing local synthesis of synaptic plasticity-related molecules such as α-CAMKII and GluR1 subunits of AMPARs. Specifically, our findings that E2 induces GluR1 synthesis in the dendrites

of hippocampal neurons elucidates a potential mechanism that E2 induced signaling via GluR1 synthesis links structural and functional plasticity at excitatory synapses.

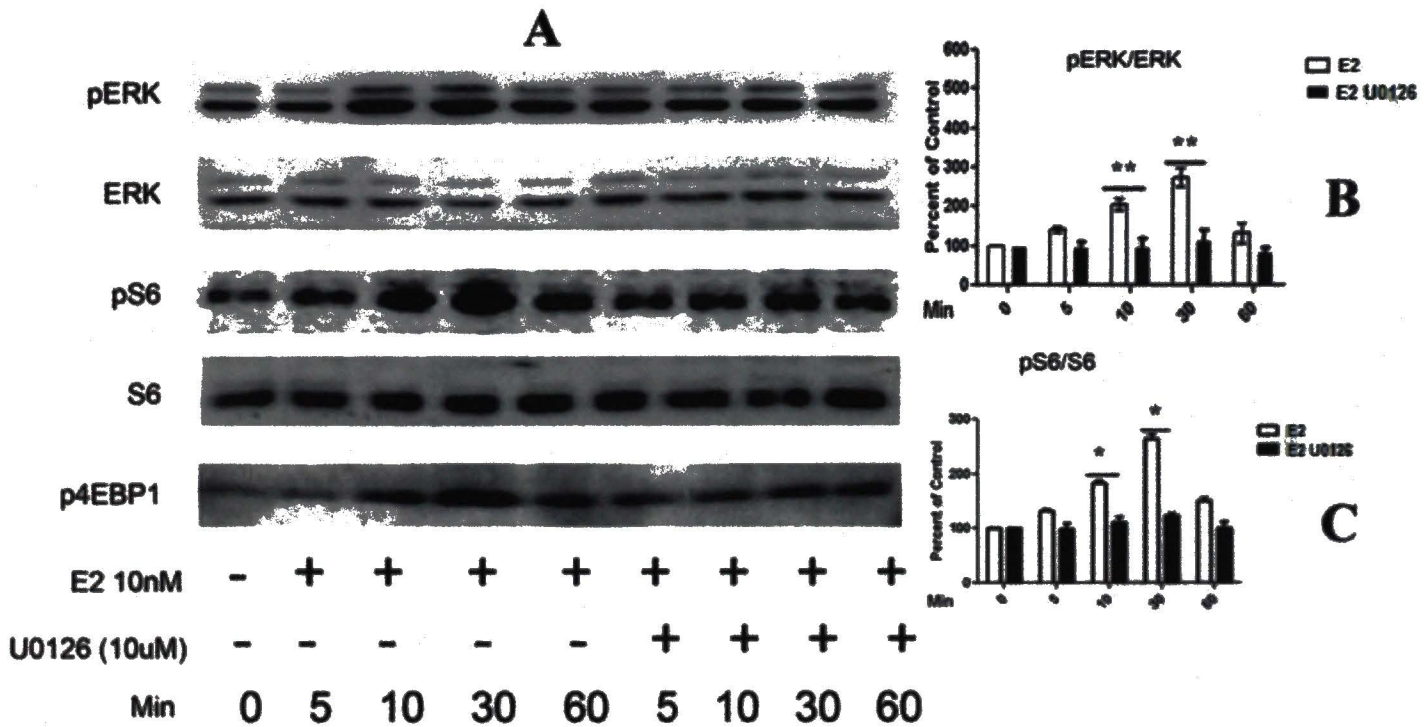
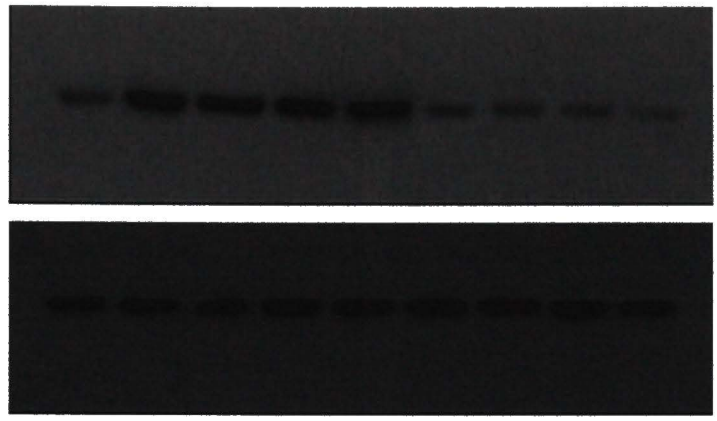


Figure 1: E2-induced ERK signaling pathway modulates the phosphorylation state of translation initiation factors. A) E2 treatment enhances and U0126 inhibits phosphorylation of ERK, S6, and 4EBP1 by E2. B) and C) Quantification of normalized labels of pERK and pS6. Two- way ANOVA was used to compare the data (*P<0.05, **P<0.01, n=3).



Ly29402 (10 μM)	-	-	-	-	-	-	-	-	+	PI3K inhibitor
Rapamycin (100 nM)	-	-	-	-	-	-	-	+	-	mTOR inhibitor
PD98059 (40 μM)	-	-	-	-	-	-	+	-	-	ERK/MAPK inhibitor
U0126 (20 μM)	-	-	-	-	-	+	-	-	-	ERK/MAPK inhibitor
E2 (10 nM)	-	+	-	-	-	+	+	+	+	
ZYC3 (10 nM)	-	-	+	-	-	-	-	-	-	non-feminizing estrogen derivative
ZYC5 (10 nM)	-	-	-	+	-	-	-	-	-	non-feminizing estrogen derivative
ZYC26 (10 nM)	-	-	-	-	+	-	-	-	-	non-feminizing estrogen derivative

Figure 2: E2 and non-feminizing derivatives of E2

induce phosphorylation of S6 by activating ERK and PI3K

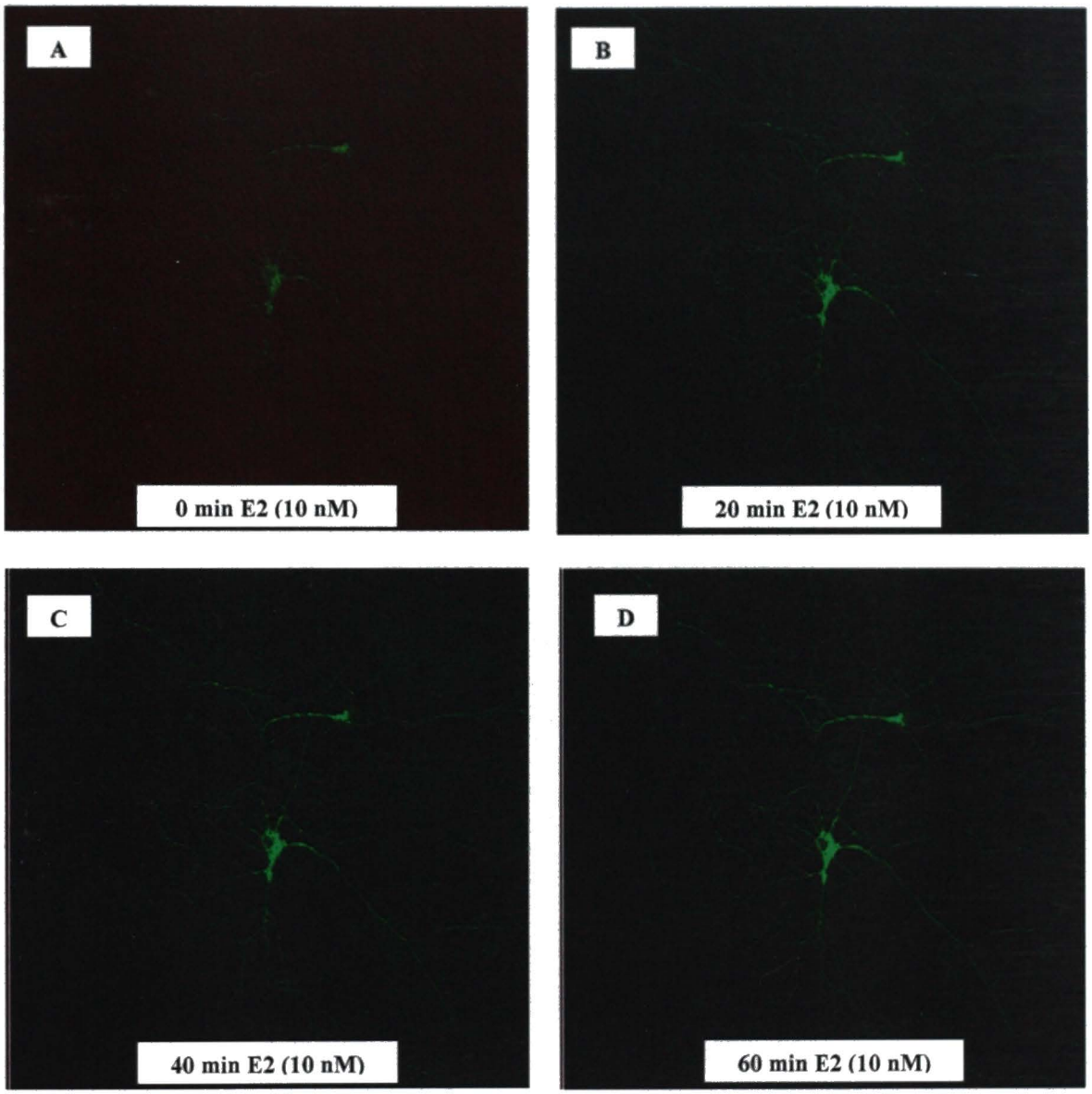


Figure 3

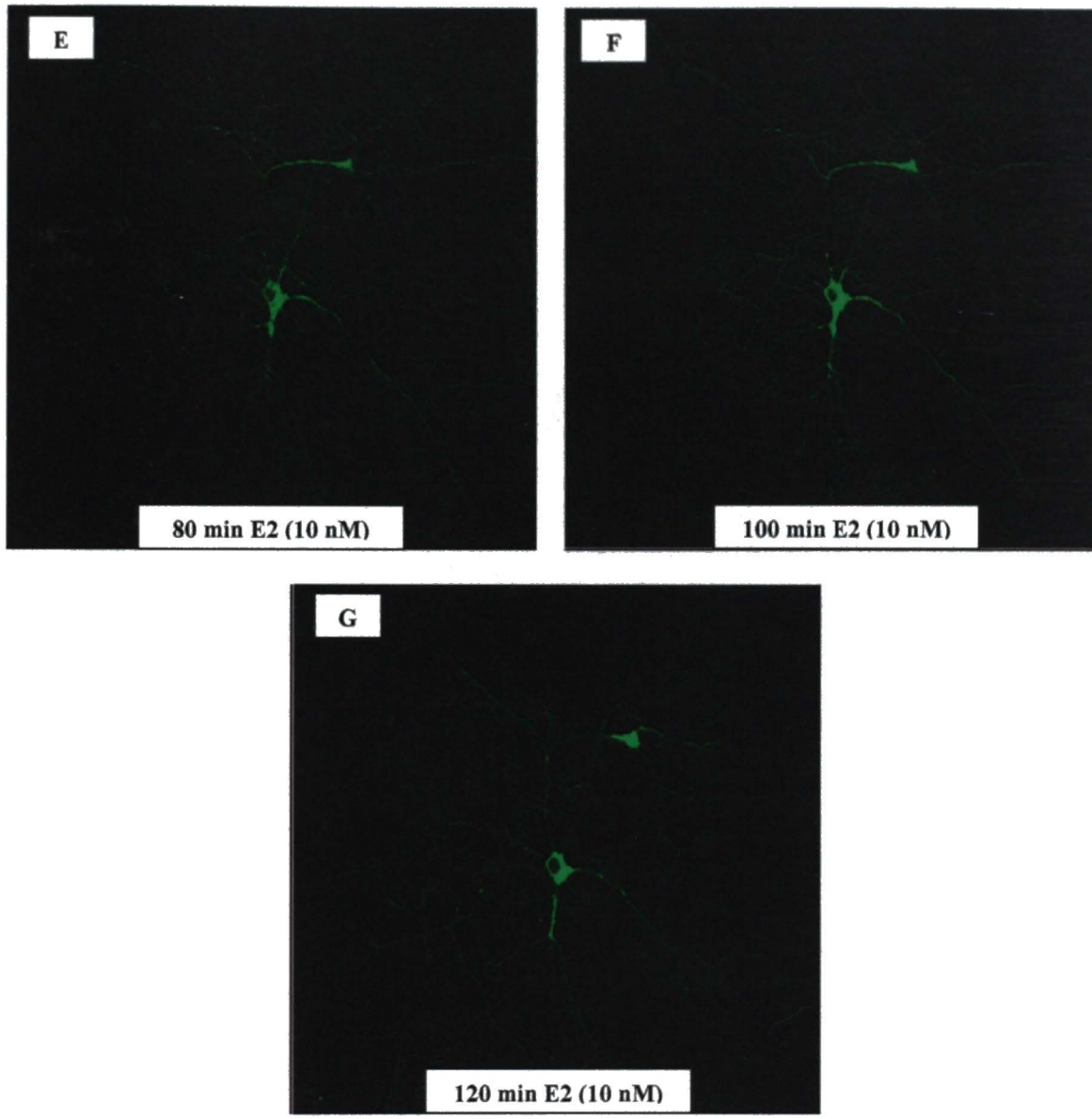


Figure 3: E2 stimulates protein synthesis of the GFP reporter in hippocampal neurons. A-G) Repeated images of E2 (10 nM) treated neurons. E2 was added immediately after the 0 min image was acquired. The E2 treated neuron showed increased fluorescence in the soma as well as the dendrites as time lapsed.

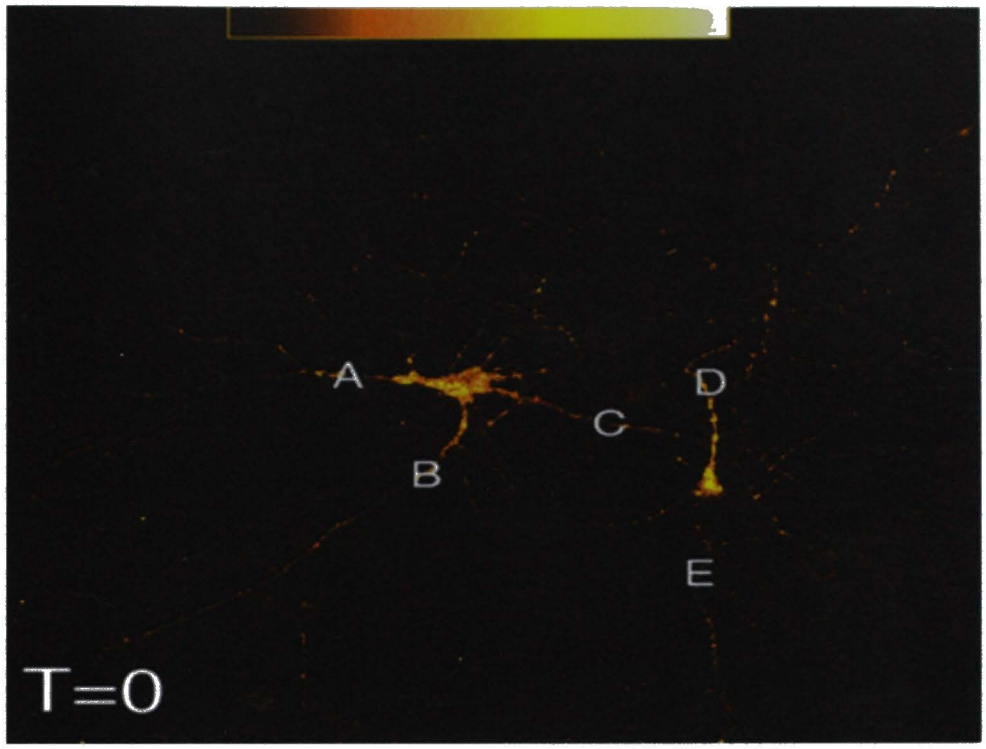
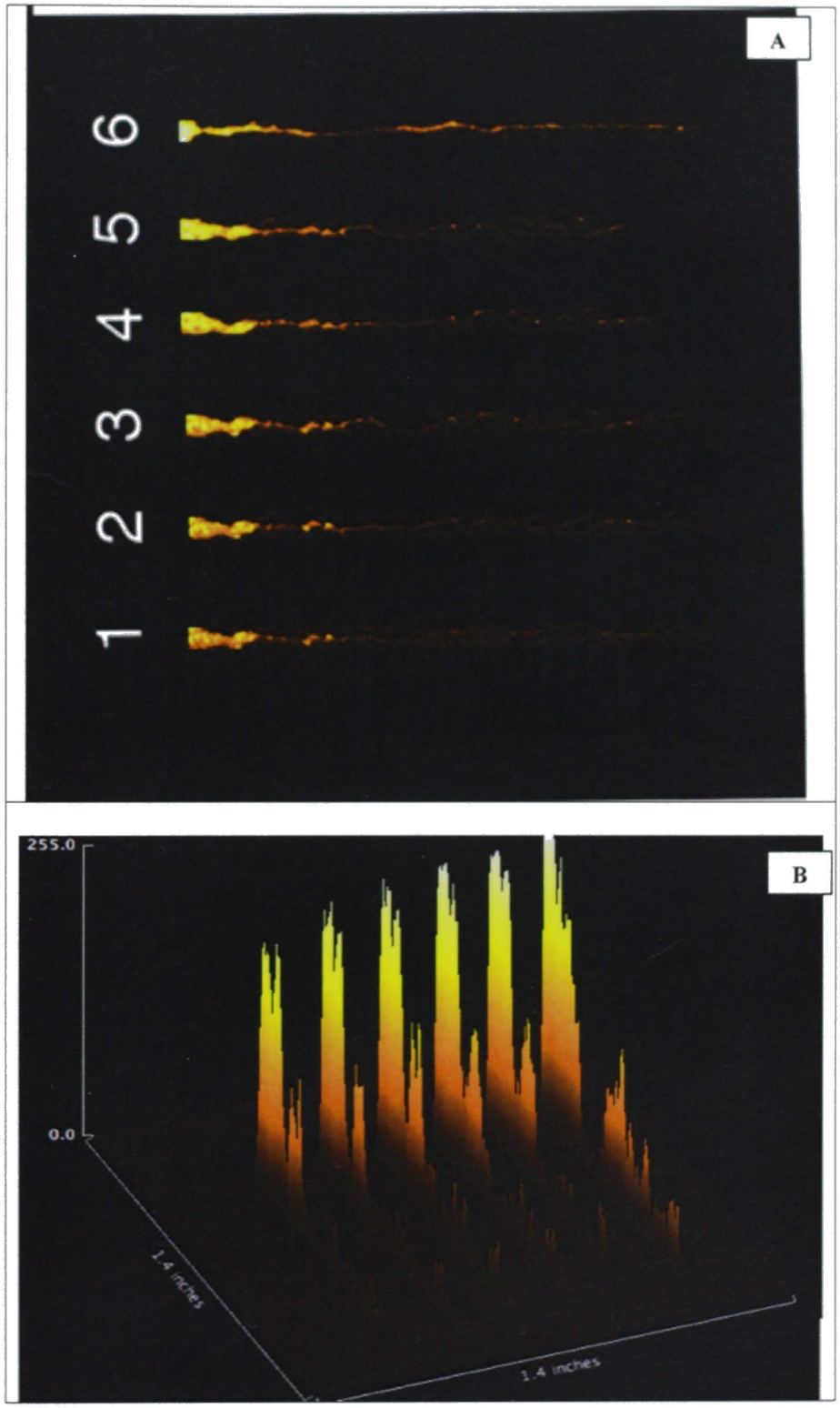


Figure 4: Labeling of dendrites expressing GFP reporter for constructing a time lapse collage as shown in Figure 5.


A
1 2 3 4 5 6
B
255.0
0.0
1.4 inches
1.4 inches

Figures 5: A) A time lapse collage of the dendrite (straightened) from the initiation of E2 treatment to 120 mins. at 20 min. intervals.

B) Relative fluorescence intensity (reporter expression) is indicated by the color and height of the pixels. A spatially specific and progressive increase in dendritic reporter expression is evident after E2 treatment.

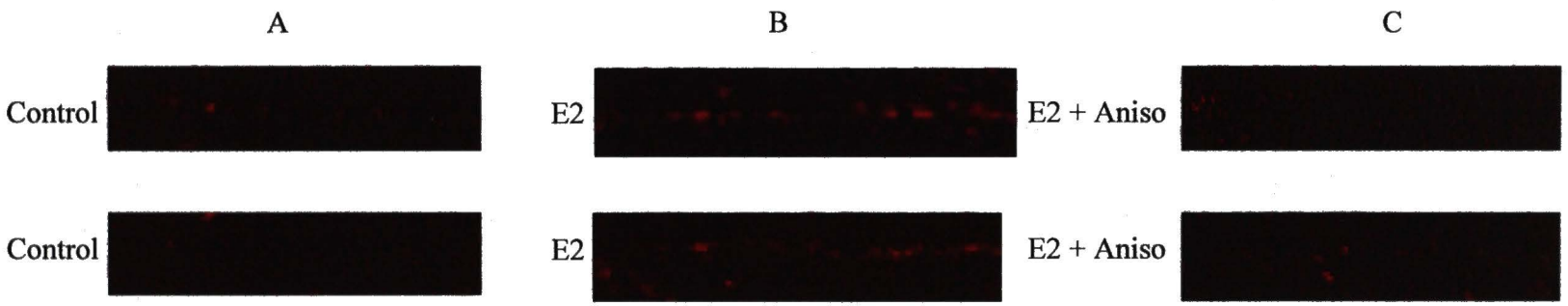


Figure 6: E2 induces GluR1 synthesis in dendrites of hippocampal neurons. A) Surface expression of GluR1 in the dendrites of hippocampal neurons (DMSO control). B) E2 (10 nM) treatment for 30 mins. C) E2 + Anisomycin (10 μg/ml) treatment for 30 mins.

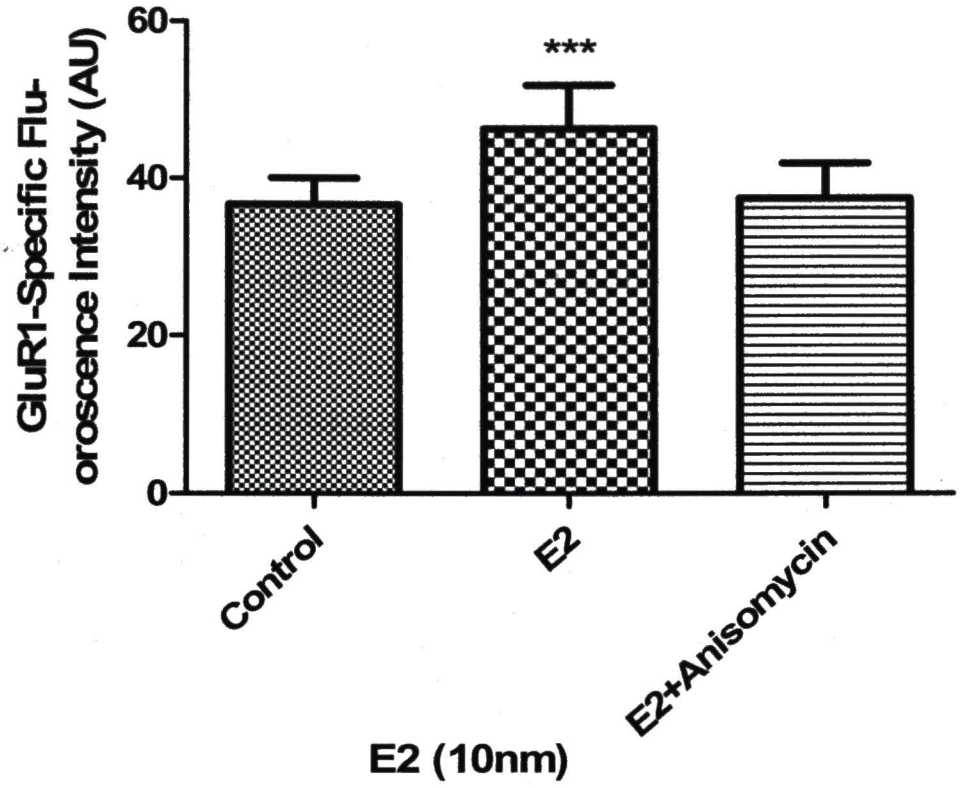


Figure 7: Quantitative analysis of GluR1 synthesis in hippocampal dendrites.

*** $p < 0.05$

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