Estrogen-induced Signaling Links Structural and Functional Synaptic Plasticity

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It is well documented that of its many roles, estrogen can acutely alter the intrinsic and synaptic physiology of neuronal circuits in various regions of the brain. However, the molecular and cellular mechanisms by which estrogen couples electrophysiology to plasticity and memory are still not fully understood. Our data suggests a new possible mechanism by which estrogen, via L-type voltage-gated calcium channel (L-type VGCC) potentiation, modulates memory related synaptic plasticity.

The rapid onset of 17β-estradiol (E2) action (less than one second) supports the hypothesis that E2 directly interacts with the channel protein. Several techniques allowed us to confirm that not only does E2 bind with high affinity to the L-type VGCC, but that it binds at a domain that overlaps with the dihydropyridine (DHP) site.

Further, to determine whether E2-induced biochemical signaling mechanistically links synaptic plasticity, we studied the phosphorylation patterns of structural and functional plasticity related proteins (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors [AMPAR], AMPA-type glutamate receptor subunit 1 [GluR1], calcium/calmodulin-dependent protein kinase II [CaMKII], and extracellular signal-related kinase [ERK]). E2 rapidly increased phosphorylation of CaMKII, ERK, and AMPAR in primary cortical neurons and in vivo in the cortex. The CaMKII inhibitor (KN-93) decreased phosphorylation levels of GluR1 in primary cortical neurons. We also
determined that soluble amyloid-beta (Aβ)_{1-42} oligomers abrogated, while E2 ameliorated phosphorylation of GluR1 at its CaMKII site. Aβ treatment also inhibited GluR1 trafficking, but E2 prevented this inhibition. Due to our observation that E2 treatment rapidly increased spine number and ameliorated Aβ-induced spine loss, we concluded that estrogen-induced signaling does in fact mechanistically link structural and functional plasticity.

In comparison with the cortical data (in vitro and in vivo), we found that E2 treatment in hippocampal slice culture ameliorated Aβ oligomer-induced inhibition of CaMKII and AMPAR phosphorylation, reduction of dendritic spine density, and abnormalities in LTP-induced spine growth. Taken together, these results suggest that acute estrogen treatment has the potential to prevent Aβ oligomer-induced synaptic dysfunction.
ESTROGEN-INDUCED SIGNALING LINKS
STRUCTURAL AND FUNCTIONAL
SYNAPTIC PLASTICITY

DISSERTATION

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

DOCTOR OF PHILOSOPHY

By

Shaun M. Logan, B.S., M.S.
Fort Worth, Texas
May 2009
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<td>AAV2</td>
<td>adeno-associated virus 2</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid-beta</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<td>AMPAR</td>
<td>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>Ca²⁺</td>
<td>calcium</td>
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<td>α-CaMKII</td>
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<td>cLTP</td>
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<td>embryonic day 18</td>
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<tr>
<td>E2</td>
<td>17β-estradiol</td>
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<td>ERα</td>
<td>estrogen receptor alpha</td>
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<tr>
<td>ERβ</td>
<td>estrogen receptor beta</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>Abbreviation</td>
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<tr>
<td>GluR1</td>
<td>AMPA-type glutamate receptor subunit 1</td>
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<tr>
<td>LTD</td>
<td>long-term depression</td>
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<td>LTP</td>
<td>long-term potentiation</td>
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<tr>
<td>L-type VGCC</td>
<td>L-type voltage-gated calcium channels</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MEK</td>
<td>mitogen activated protein kinase kinase</td>
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<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
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<td>PBS</td>
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<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>WHIMS</td>
<td>women health initiative memory studies</td>
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CHAPTER I. INTRODUCTION

Estrogens and Function

Estrogens are a group of steroid compounds named for their importance in the estrous cycle. While estrogens are present in both men and women, they are usually present at significantly higher levels in women of reproductive age. The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the predominant form in nonpregnant females, estrone is produced during menopause, and estriol is the primary estrogen during pregnancy. Among these, estradiol is the most potent. In the body these are all produced from androgens through actions of enzymes. Estrogens were first described as the primary female sex hormone for reproductive development and function, but over time their function in mammals has proven to be much more diverse.

Excitability

Estrogen, usually in the form 17β-estradiol (E2), has been shown to acutely alter the intrinsic excitability of neurons in a wide variety of brain regions including the hypothalamus (Kelly et al., 1977), amygdala (Nabekura et al., 1986), striatum (Mermelstein et al., 1996), cerebellum (Smith et al., 1988), and hippocampus (Wong et al., 1991). These experiments were performed both in vitro and in vivo, and demonstrated that neuronal firing rates were rapidly altered. Estrogen treatment also modulates potassium (K⁺) currents that control the resting membrane potential, or limit the occurrence of action potentials (Wong et al., 1991).
**Synaptic Physiology and Plasticity**

In addition to its effects on membrane excitability, E2 also acutely modulates synaptic physiology in the hippocampus. Application of 100 pM E2 to hippocampal slices increased dendritic depolarization as measured by the field excitatory post synaptic potential (fEPSP) [Teyler et al., 1980]. In addition, E2 was shown to increase dendritic depolarization and long-term potentiation (LTP) in CA1 slices from male rats (Foy et al., 1999; Bi et al., 2000; Fugger et al., 2001), and estrogen receptor alpha (ERα) knock-out male and female mice (Fugger et al., 2001). Recently, this effect has been demonstrated in the hippocampal CA3 and dentate gyrus regions (Kim et al., 2006). In addition to its effects on baseline synaptic responses, E2 can acutely increase the capacity for synaptic plasticity in the CA1 region. LTP is a well established model of activity dependent enhancement in synaptic efficacy that is thought to underlie learning and memory formation. LTP is generally induced using brief, high frequency trains of presynaptic stimuli delivered to hippocampal slices or in vivo.

The initiation of LTP at excitatory synapses in the CA1 region requires both N-methyl-D-aspartate receptor (NMDAR) and L-type voltage-gated calcium channels (L-type VGCC) activation (Magee et al., 1997). On the other hand, the resulting potentiation of synaptic strength occurs largely through modifications in the number and/or function (phosphorylation, insertion, or endocytosis) of alpha-aminooxyacid-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs). The degree of potentiation is related to the level of post-synaptic calcium ion (Malenka et al., 1988). High frequency trains delivered in the presence of 100 pM E2 produced greater LTP when compared with control (Foy et
al., 1999). An increase in baseline synaptic transmission and LTP, both of which were induced by E2, were blocked by tyrosine kinase and mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) inhibitors, which also blocked E2 induced phosphorylation of NMDARs (Bi et al., 2000). Collectively, these findings demonstrate that short-term treatment of E2 in hippocampal neurons leads to enhancement of neuronal excitability, glutamatergic synaptic transmission, and synaptic plasticity that could acutely facilitate hippocampal dependent memory.

**Acute Effects of Estrogen on Memory in Animals**

It has been reported that acute effects of E2 on intrinsic and/or synaptic physiology in the hippocampus contribute to improved hippocampal dependent memory. For example, both CA1 pyramidal cell reduction of after hyperpolarization (AHP) [Weiss et al., 2005] and increased capacity for LTP in the CA1 region (Cordoba-Montoya et al., 1997; Good et al., 1999; Sandstrom et al., 2001; Daniel et al., 2001) correlate positively with performance in memory tasks that are dependent upon the hippocampus. With the use of a Morris water maze, it was shown that short-term effects of E2 on retention require hippocampal-dependent spatial memory. In these studies, the authors have also demonstrated that intrahippocampal injection given immediately following training, improved the rat’s ability to find the platform location compared to when E2 is given 2 hours after training (Packard et al., 1996; Packard et al., 1997). Two additional studies have also confirmed time limited effects of various estrogens (17α, 17β, and synthetic estrogens-diethyl bystrol [DES]) [Luine et al., 2003] in improving memory; these studies used object recognition, place memory, or inhibitory avoidance task. These tasks have a
spatial component and are dependent upon the hippocampus. Together, these behavioral studies show that E2 can act within a relatively short time to facilitate memory processes that involve the hippocampus. In contrast, the time course of the short-term effects of E2 in memory studies (less than 2 hours) is longer than that of electrophysiological studies (several minutes), and could involve genomic effects. The results are consistent with the possibility that acute E2-induced excitability and/or excitatory synaptic transmission, and LTP in the hippocampus, contribute to improved memory retention.

**Estrogen Levels, Brain Activity, and Estrogen Replacement Therapy in Humans**

Various experimental studies have shown that the concentration of E2 in the blood has an influence on cognitive abilities (Dietrich et al., 2001). The variation of performance during neuropsychological tasks in different sexes and during different phases of the mirtal cycle, correlates with the cortical activation pattern as measured by functional magnetic resonance image (MRI) [Amin et al., 2006]. Low dose E2 treatment in postmenopausal 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 sub-cortical and cortical brain regions (Stevens et al., 2005). E2 replacement also enhances memory and cognition in women who have undergone surgical menopause (Sherwin et al., 1988; Philllips et al., 1992; Sherwin et al., 2003). E2 treatment improved cognitive function in women diagnosed with Alzheimer’s disease (AD) in several clinical trials (Fillit et al., 1986; Honjo et al., 1989; Ohkura et al.,
1994). The results of recent Women Health Initiative Memory Studies (WHIMS) demonstrated a deleterious effect of combined conjugated equine estrogen with respect to risk for AD or cardiovascular dysfunction (Shumaker et al., 2004; Shumaker et al., 2003). Therefore, WHIMS findings stand in contrast with a considerable body of literature. The majority of literature suggests that exogenous estrogen helps to protect cognitive function in postmenopausal women. It is possible that the negative health risks identified in WHIMS may be the result of the timing, dose, and formulation of dosing interventions. In observational studies, unlike the WHIMS randomized trials, the results may have been somewhat biased due to the health of the subjects. A possible explanation for inconsistencies between prior studies and WHIMS is that estrogen has been reported to enhance verbal memory with no effect on other domains of cognition (Sherwin et al., 2003). Although the Modified Mini Mental State Examination (3MSE) is a reliable and valid measure of global cognitive function, it does not capture performance in individual domains of cognition. It remains possible that differential, beneficial, or detrimental effects of estrogen on specific cognitive function (verbal memory) could not be detected by 3MSE used in WHIMS. The timing theory may be related to a critical period in which hormone therapy must be initiated to protect cognitive function. Laboratory animal models suggest that estrogen treatment 3 months and not 10 months after ovariectomy produces memory performance similar to that of young control animals (Gibbs et al., 1999; Rapp et al., 2003). Future research will help us to better understand and isolate the mechanism of estrogen’s beneficial effects, so that a more informed judgment can be made regarding the risks and benefits to patients undergoing hormone therapy.
Mechanisms Involved in Estrogen Action

Estrogen acts in different regions in a variety of cells; one being in the nucleus and the other being at the membrane. Nuclear actions are mediated by ERα and ERβ, and involve gene transcription through ERs binding to their respective estrogen response element (ERE). Estrogen responsive genes include epidermal growth factor (EGF), brain derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (Miranda et al., 1996; Sohrabji et al., 1994; Toran-Allerand CD et al., 1992). The possibility of extra nuclear ERs in neurons was first suggested by Blaustein et al., 1992, who showed ERα immunoreactivity in dendrites and axonal boutons in the hypothalamus. More recently Milner et al., 2001, have shown immunoreactivity for ERα and ERβ (Milner et al., 2005) in glia and in some axons, dendrites, and dendritic spines in the hippocampus. In addition to extra nuclear localization of the classical nuclear ER proteins, a third extra nuclear ER (ER-X) has also been reported (Toran-Allerand et al., 2002). Although ER-X has not yet been cloned, it has homology with ERα and can be recognized by some c-terminal ERα antagonist. ER-X is present primarily in caveolar-like domains of neuronal plasma membranes, and can mediate activation of the MAPK/ERK signaling cascade by 17α-estradiol and E2 (Toran-Allerand et al., 2002). Evidence accumulated over the past decade has demonstrated that E2 rapidly modulates neuronal excitability an effect that occurs via action of cellular signaling cascades (Singh et al., 1999; Bi et al., 2000; Bi et al., 2003; Honda et al., 2000). These rapid effects of E2 which occur too fast to be mediated by the classic genomic pathway are generally assumed to be initiated at the cell membrane, due to the existence of specific membrane associated receptors. E2 binding to
membrane localized receptors has been shown to activate multiple intracellular cascades, including MAPK/ERK and phosphoinositide 3-kinase PI3K/Akt pathways (Kow et al., 2004; Marino et al., 2006; Vasudevan et al., 2005). The detailed mechanism by which E2 modulates neuroprotection, synaptic plasticity, and cognitive functions is still not understood. It has been generally proposed that this effect requires a dual mode of cellular regulation, one involving signaling cascades down stream of a membrane-bound receptor and the other involving regulation of gene expression.

**Role of Calcium as a Second Messenger in Neuronal Survival and Synaptic Plasticity**

Calcium is a second messenger, which can trigger the modification of synaptic efficacy. Long-term changes in synapses are thought to be the cellular basis of information storage and memory formation. Protocols used to induce plasticity, like repetitive low frequency synaptic stimulation, also induce the elevation of post synaptic intracellular calcium. Calcium influx acts on down-stream metabolic cascades that are responsible for eventual modification of synaptic efficacy. Calcium influx through L-type VGCC (Westenbroek et al., 1990; Magee and Johnston, 1995) and glutamate-gated ion channels (Bekkers and Stevens, 1989; Spruston and Sakmann, 1995) located in the post synaptic membrane of excitatory synapses, results in a transient elevation of intracellular calcium during neuronal activity (Markram and Sakmann, 1994). The elevated level of intracellular calcium can activate numerous kinases (alpha-calcium/calmodulin-dependent protein kinase [α-CaMKII], CaMKIV, protein kinase A [PKA], protein kinase C [PKC], MAPK/ERK, and PI3K/Akt) and phosphatases (protein phosphatase 1 [PP1],
protein phosphatase 2A [PP2A], and protein phosphatase 2B [PP2B]), which respectively phosphorylate or dephosphorylate ion channels, transcription factors, and other proteins that are involved in neuronal survival, synaptic plasticity, and memory formation (Lee et al., 2000; Lisman et al., 1989; Nevian and Sakmann, 2006). Hippocampal synaptic plasticity and various forms of learning related behavior critically depend on the MAPK/ERK cascade and the resulting stimulation of gene expression, and also de novo protein synthesis (specifically cyclic adenosine monophosphate (cAMP) response element-binding [CREB] protein) [English et al., 1997; Atkins et al., 1998; Hardingham et al., 2001; Kandel et al., 2001; Wu et al., 2001; Pittenger et al., 2002; Thomas and Huganir, 2004]. Induction of LTP at schaffer collateral/CA1 synapses, as well as the activation of ERK/CREB pathway in hippocampal CA1 neurons, requires an increase in post synaptic intracellular calcium concentration (Shaywitz and Greenberg, 1999; Kandel, 2001). Calcium influx via L-type VGCC can support a form of NMDAR-dependent (Magee et al., 1997), or -independent LTP (Morgan and Teyler, 1999), and sustained CREB phosphorylation with subsequent action of cAMP response element (CRE)-gene expression in hippocampal neurons (Dolmetsch et al., 2001). Particularly CCAAT-enhancer binding protein beta (C/EBPβ) which is down-stream from CRE is increasingly induced in response to calcium and cAMP signals (Alberini et al., 1994). Synaptic and extra synaptic localizations of L-type VGCC correspond to putative roles of L-type calcium currents in synaptic modulation and the propagation of dendritic calcium spikes respectively. Recently, the function of L-type VGCC in spatial learning, synaptic plasticity, and triggering of learning associated biochemical processes were evaluated in a
transgenic mouse with an inactive Ca\textsubscript{v}1.2 (α1\textsubscript{c}) gene in the hippocampus and neocortex (Moosmang et al., 2005). This study showed selective loss of protein synthesis-dependent, but NMDAR-independent LTP, severe impairment in hippocampal-dependent spatial memory, loss of activation of MAPK pathway, and repressed CRE-dependent transcription in hippocampal neurons. Below are several different schematics which all depict how calcium influx via NMDAR and/or L-type VGCC leads to the activation of kinases and transcription factors, resulting in the expression of genes which induce LTP:

**Figure 1**

**Activation of ERK by synaptic signaling and downstream targets**

Calcium influx through NMDAR and/or L-type VGCC triggers an increase in the levels of Ras-guanosine triphosphate (GTP), which leads to activation of Raf, mitogen activated protein kinase/ERK kinase (MEK), and ERK, allowing phosphorylation of cytosolic and
nuclear substrates. Following its activation, ERK phosphorylates membrane targets such as L-type VGCC and K⁺ channels, and also extranuclear targets (ribosomal protein S6 kinases [RSKs]). Activated ERK and RSK, translocate to the nucleus where ERK activated/phosphorylates the constitutively nuclear mitogen-and stress-activated kinases (MSKs). In the nucleus, ERK, RSKs, and MSKs phosphorylate transcription factor substrates (CREB).

**Figure 2**

**LTP is initiated in the postsynaptic cell and requires NMDA receptors**

α-CaMKII is one of the best candidates for being a molecular component of the learning and memory machinery in the brain. It is present in abundance at the synapse, and its kinase properties and responsiveness to calcium influx, either through NMDAR or L-type VGCC, fits a model whereby calcium currents activate the kinase, which in turn leads to changes in synaptic efficacy. Recent cell biological, genetic, and physiological analyses suggest that one of the cellular explanations for LTP and α-CaMKII function might be the trafficking of AMPAR to synapses in response to neuronal activity (as shown in diagram).
As shown in the diagram, heightened synaptic activity in hippocampal neurons induces calcium influx through NMDAR and L-type VGCC (CaCh respectively). The synaptic activity in hippocampal neurons also induces cAMP signaling through modulatory inputs like dopamine via its receptors. cAMP and calcium signals converge in the nucleus and activate CREB. Activated CREB then induces the expression of C/EBPβ. CaMKIV and PKA phosphorylates C/EBPβ, C/EBP delta (δ), and transactivates effector genes for long-term memory.

**Triggering LTP**

CaMKII is a key component of the molecular machinery for triggering LTP. CaMKII undergoes autophosphorylation after the triggering of LTP (Barria et al., 1997), and it has been shown that LTP induction was prevented in both knock-out mice lacking a critical CaMKII subunit (Silva et al., 1992), and knock-in animals in which endogenous
CaMKII was replaced with a form lacking the autophosphorylation site (Giese et al., 1998).

Several other kinases have been implicated in the triggering of LTP. Activation of cAMP-dependent PKA has been suggested to boost the activity of CaMKII indirectly by decreasing competing protein phosphatase activity (Blitzer et al., 1998). The MAPK/ERK pathway has also been suggested to be important for LTP, as well as hippocampal-dependent learning and memory (Thomas and Huganir, 2004). Also, it has been demonstrated that the atypical PKC isozyme, PKM ζ, is rapidly expressed upon induction of LTP and recent studies have implicated PKMζ in the maintenance of LTP both in hippocampal slices and in vivo (Pastalkova et al., 2006; Serrano et al., 2005).

**Expression of LTP**

The major mechanism of expression of LTP at hippocampal CA1 synapses involves an increase in the number of AMPARs within the postsynaptic density, driven through activity-dependent changes in AMPAR trafficking (Malenka and Nicoll, 1999; Bredt and Nicoll, 2003). Recent studies indicate that LTP and long-term depression (LTD) may be expressed, in part, by regulation of AMPAR function (Song and Huganir, 2002). The regulation of AMPAR function occurs through two distinct but interrelated mechanisms: 1) modulation of ion channel properties of the receptor, and 2) regulation of the synaptic targeting of the receptor (Benke et al., 1998; Hayashi et al., 2000; Shi et al., 2001). Both of these processes are regulated by protein phosphorylation of the receptor (Song and Huganir, 2002). LTP is associated with an increase in phosphorylation of
AMPAR (GluR1 subunit) [Lee et al., 2000], and LTD is correlated with a dephosphorylation of GluR1 (Kameyama et al., 1998; Lee et al., 2000). Also, it has been demonstrated that mice with knock-in mutations at GluR1 phosphorylation sites show deficits in LTD and LTP, and have memory deficits in hippocampal-dependent spatial learning tasks (Lee et al., 2003). The depiction in figure 4 represents the bidirectional changes in AMPAR phosphorylation and NMDAR-dependent synaptic plasticity:

Figure 4

![Diagram showing bidirectional changes in AMPAR phosphorylation and NMDAR-dependent synaptic plasticity](image)

When a high frequency stimulation (HFS) is delivered to naïve synapses, CaMKII is preferentially activated, and this in-turn leads to the increase in phosphorylation of GluR1 at serine 831 (CaMKII site), resulting in LTP. On the other hand, low frequency stimulation (LFS) delivered to naïve synapses results in the activation of protein phosphatases (PP1/2A), which dephosphorylate serine 845 (PKA site).

**Maintaining LTP**

Mechanisms which allow LTP to persist for hours, days, or even longer, all depend upon new protein synthesis (Reymann and Frey, 2007). Maintenance of LTP
requires both local dendritic protein synthesis (which supplies needed components to the synapse) [Sutton and Schuman, 2006] and transcription in the nucleus. It has been suggested that signaling to the nucleus which is required for long-lasting LTP depends on a number of protein kinases including PKA, CaMKIV, and MAPK/ERK, which activate key transcription factors that include CREB and immediate-early genes (c-Fos and Zif268/Egr-1) [Thomas and Huganir, 2004]. These transcriptional complexes presumably promote expression of effector genes which are required for maintaining the enhancement of synaptic strength. Pang et al., 2004, reported that mature BDNF is a key protein synthesis product for late-phase LTP. Moreover, application of mature BDNF was sufficient in rescuing late-phase LTP when protein synthesis was inhibited. Another mechanism for long-term maintenance of LTP is the structural remodeling of potentiated synapses (Luscher et al., 2000).

Spines come in a variety of shapes and sizes, and can undergo rapid shape changes that are influenced by neuronal activity (Yuste and Bonhoeffer, 2001). Morphological changes that have been reported to accompany LTP include growth of new dendritic spines, enlargement of pre-existing spines and their post synaptic densities (PSDs), and the splitting of spines and single PSDs into two functional synapses (Matsuzaki et al., 2004; Nagerl et al., 2004; Okamoto et al., 2004; Sorra et al., 1998). It has been suggested that during LTP, recycling endosomes contribute AMPAR subunits to the synapse, as well as lipids and constituents which enlarge the synapse (Lisman and Zhabotinsky, 2001; Luscher et al., 2000; Park et al., 2004; Park et al., 2006). The
schematic in figure 5 shows LTP-induced AMPAR insertion and translocation of recycling endosomes into spines.

**Figure 5**

LTP-induced AMPAR insertion and membrane addition from recycling endosomes correlates with the scaling of spine size. When recycling to the exocytic zone (ExoZ) and endocytic zone (EZ) is decreased this results in spine loss.

**Non-fibrillar Soluble Amyloid-beta (Aβ) Oligomers, Synaptic Dysfunction, and AD**

The Aβ hypothesis proposes that continuous disruption of normal synaptic physiology by soluble Aβ oligomers contributes to the development of AD. Aβ oligomer-induced aberrations in synapse composition, shape, and density, provide a molecular basis for the loss of connectivity in neural circuits of the AD brain. Naturally secreted, small, soluble, oligomers of human Aβ (trimers) that were isolated from cultured cells inhibited hippocampal LTP in vitro and in vivo, and transiently impaired the recall of a memory task in rats (Walsh et al., 2002). Using the chemically-induced LTP (cLTP) paradigm on neuronal cultures, treatment with soluble oligomers resulted in inhibition of cLTP-induced activation of synaptic plasticity related kinases (MAPK/ERK, CaMKII, and Akt) [Townsend et al., 2007]. Furthermore, soluble Aβ oligomers (dimers) isolated
directly from the cerebral cortex of subjects with AD effectively inhibited LTP, enhanced LTD, and reduced dendritic spine density in a normal rodent hippocampus (Shankar et al., 2008). In the same study, Aβ dimers disrupted the memory of learned behavior in normal rats.

Even though build up of Aβ oligomers has been identified time and time again as one of the culprits that causes AD, it is still unknown whether specific receptors mediate the adverse effects seen with the disease. Just recently, Laurén et al., 2009, showed that the prion protein (PrP, maintains white matter) might mediate the pathogenic effects of Aβ oligomers. They found that Aβ oligomers inhibited LTP in hippocampal slices from normal mice, but not in hippocampal slices from mice lacking the cellular form of the prion protein. Also, LTP was not affected by Aβ oligomers in hippocampal slices from normal mice where Aβ was not allowed to interact with the cellular form of the prion protein. Because PrP ablation only reduced the binding of Aβ oligomers to neurons by 50%, the existence of other receptors for Aβ oligomers can not be excluded.

Transgenic AD Mouse Model

The molecular, cellular, and pathological changes which trigger the onset of cognitive decline in the AD brain are presently unknown and are intractable problems to address in humans. Hence, animal models remain invaluable for identifying the molecular markers which trigger the onset of AD related cognitive decline. Toward this end, various laboratories have generated transgenic mouse models of AD that mimic many critical aspects of AD pathology (Savonenko et al., 2005; Jankowsky et al., 2002; Holcomb et al., 1998; King et al., 1999; McGowan et al., 1999). Clearance of the
intraneuronal Aβ pathology by immune therapy, rescues the early cognitive deficits seen in hippocampal-dependent memory tasks (Morgan et al., 2000). Thus, this study strongly indicates that intraneuronal Aβ contributes to the onset of AD. Using transgenic AD mice, several preclinical studies demonstrated that Aβ accumulation and hippocampal-dependent memory impairment can be effectively attenuated not only by E2, but also specific estrogen receptor modulators (SERMs) [Carrol et al., 2007; Levin-Allerhand et al., 2002]. Also, brain specific aromatase gene knock-out in transgenic AD studies has demonstrated that E2 brain levels are critical to Aβ regulation in these mice (Yue et al., 2005).

**Estrogens and Transgenic AD Mice**

The effects of ovariectomy and E2 on spatial learning and memory, hippocampal Aβ levels, and amyloid plaque counts were investigated in amyloid precursor protein and presenilin 1 double knock-in transgenic mice (Heikkinen et al., 2004). Also in this study, E2 treatment in ovariectomized mice increased the number of correct choices during a position-discrimination task in the T-maze, and slightly improved their performance during a win-stay task in the radial arm maze (RAM). Furthermore, neither ovariectomy nor E2 treatment had an effect on hippocampal amyloid accumulation in ovariectomized transgenic AD mice. These results show that E2 treatment in a transgenic mouse model of AD improved performance during the same learning and memory task as when E2 was administered to normal mice. However, the E2 effects in these mice appeared to be unrelated to Aβ-mediated cognitive deficits. Thus, it is not clear if E2 treatment decreases the risk or alleviates the symptoms of AD by inhibiting the accumulation of Aβ or
formation of Aβ plaques. In a triple transgenic AD mouse model (Oddo et al., 2003), the AD like neuropathology was apparent within three months of age and progressively increased through twelve months of age in gonad intact female transgenic AD mice; the time course was paralleled with behavioral impairment (Carroll et al., 2007). Ovariectomy-induced depletion of sex steroid hormones in adult female triple transgenic AD mice significantly increased Aβ accumulation and worsened memory performance. Treatment of ovariectomized triple transgenic AD mice with estrogen or progesterone prevented these effects. When estrogen and progesterone were administered in combination, progesterone blocked the beneficial effects of estrogen on Aβ accumulation but not on behavioral performance. Interestingly, progesterone significantly reduced tau hyperphosphorylation when administered alone and in combination with estrogen. Collectively, these results demonstrate that estrogen and progesterone independently and interactively regulate AD like neuropathology, and suggest that an optimized hormone therapy (HT) might be useful in reducing the risk of AD in postmenopausal women.

Despite its protective effect against AD, E2 cannot be used to treat AD patients because hormone therapy is associated with adverse effects in estrogen responsive tissues which include the breast and uterus (Espeland et al., 2004). Therefore, as an alternate to E2 therapy, SERMs were used in triple transgenic mice to maximize E2 benefits and minimize E2 risks (Carroll and Pike, 2008). Ovariectomy-induced hormone depletion in adult female triple transgenic AD mice significantly increased accumulation of Aβ protein and decreased hippocampal-dependent behavioral performance. Treatment with E2 prevented the ovariectomized-induced worsening of both pathologies. 4", 4"
Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT), an ERα agonist, treatment was similar to E2 in terms of reducing Aβ accumulation in the hippocampus and amygdala, but comparatively less effective in the frontal cortex. In contrast, (diarylpropionitrile) DPN, an ERβ agonist, did not significantly reduce Aβ accumulation in the hippocampus.

**Summary**

Estrogen acutely alters the intrinsic and synaptic physiology of neuronal circuits within minutes in various regions of the brain. Most of the electrophysiological effects of E2 in the hippocampus serve to facilitate neuronal firing, increase excitatory synaptic transmission, and increase the capacity for synaptic plasticity (LTP and LTD). In neurons the activity-dependent influx of calcium through NMDAR or L-type VGCC is coupled to persistent neuronal activity, diverse electrical activity related to the induction of LTP and LTD, and activation of kinases and transcription factors important for synaptic plasticity. The molecular and cellular mechanisms by which the acute effects of estrogen on electrophysiology are coupled to plasticity and memory are not fully understood. We hypothesized that 1) estrogen directly or indirectly by induced signaling, potentiates ion channels and 2) estrogen-induced signaling links structural and functional synaptic plasticity. The findings of this study may provide significant insight into the mechanism by which estrogen via L-type VGCC modulates the neuronal circuitry involved in memory consolidation and extinction, as well as, estrogen-induced regeneration of neurons in neurodegenerative diseases like Alzheimer’s disease.
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CHAPTER II. MOLECULAR MECHANISM BY WHICH ESTROGENS POTENTIATE L-TYPE VGCC

INTRODUCTION

A large body of evidence shows that estrogens exert multiple rapid effects on the structure and function of neurons in a variety of brain regions, including the hippocampus (McEwen, 2002). For example, estrogens rapidly potentiate kainate-induced currents in hippocampal neurons from wild-type (Gu and Moss, 1998), as well as, ERα knock-out mice (Gu et al., 1999), and induce rapid spine synapse formation in the CA1 region of the hippocampus of ovariectomized rats (MacLusky et al., 2005). Further, acute application of estrogens to hippocampal slices increases NMDAR and AMPAR transmission (Smith and McMahon, 2005), induces LTP and LTD (Good et al., 1999), and rapidly modulates neuronal excitability in the rat medial amygdale (Nabekura et al., 1986) and hippocampus (Teyler et al., 1980).

It is well known that estrogens interact with cell membrane components and initiate signaling events leading to a rise in intracellular calcium (Ca\(^{2+}\)), and activation of Src kinase, G protein-coupled receptors (GPCRs), MAPK/ERK, PI3K/Akt, PKA, and adenylyl cyclase (Lee and McEwen, 2001). The mechanism(s) by which estrogens induce these rapid and diverse effects remains largely unknown. Ca\(^{2+}\) is a second messenger, which can trigger the modification of synaptic efficacy. A plasticity induction protocol like repetitive low frequency synaptic stimulation (Mulkey and Malenka, 1992) induces
the elevation of post synaptic intracellular Ca\(^{2+}\). The level of intracellular Ca\(^{2+}\) concentration can activate numerous kinases like CaMK, PKA, PKC, MAPK/ERK, PI3K, or phosphatases (Frodin et al., 1995; Finkbeiner and Greenberg, 1998; Persaud et al., 1996; Macfarlane et al., 1997; Rhodes, 2000) which respectively phosphorylate or dephosphorylate ion channels, transcription factors, and other proteins which are involved in synaptic plasticity and memory formation. Because L-type VGCC mediated extracellular Ca\(^{2+}\) influx in neurons initiates the activation of these same signaling cascades (Morozov et al., 2003; Rusanescu et al., 1995; Dolmetsch et al., 2001; Chan et al., 2005; Vaillant et al., 1999), our laboratory hypothesized that estrogens potentiate L-type VGCC, and subsequently reported that estrogen facilitates L-type VGCC in hippocampal neurons via an ER-independent mechanism, through direct binding with a domain that overlaps the dihydropyridine (DHP) binding site. Moreover, the capacity of estrogen to potentiate specifically L-type VGCC may impart a distinctive role of estrogen in modification of synaptic efficacy.

MATERIALS AND METHODS

Expression of Ca\(^{2+}\) channels

Human embryonic kidney (HEK) 293 cells were transfected using TransIT\(^{\text{TM}}\)-293 transfection reagent (Mirus, Madison, WT). Cells were transfected with a 2:1:1 ratio of plasmid DNA comprised of neuronal, wild-type, or mutant \(\alpha_1\) (Ca\(_{1.2}\)), \(\beta_1b\), and \(\alpha_2\delta\) L-type subunits (gift from Dr. Michael E. Greenberg, Harvard University, Dolmetsch et al., 2001) and a green fluorescent protein (GFP) expression plasmid, pGFP-C1 (Clontech, Mountain View, CA), with a ratio of 10:1 channel subunits to GFP. For whole cell
binding experiments, HEK 293 cells were transfected with a 2:1:1 ratio of plasmid DNA comprised of either wild-type or mutant α1C (T1065Y), and β1b and α2δ L-type subunits.

**Whole Cell Ligand Binding Assay**

HT-22 cells, an immortalized murine hippocampal neuronal cell line, grown in Dulbecco’s modified eagle medium (DMEM) [Gibco, Carlsbad, CA], supplemented with charcoal stripped fetal bovine serum (FBS) [Hyclone, Logan, UT], were fixed with 4% paraformaldehyde at room temperature for 10 minutes (mins.) and washed three times with phosphate buffered saline (PBS). Fixed cells were blocked with 10% goat serum for 2 hours (hrs.) at room temperature, and then incubated overnight at 4°C with anti-Cav1.2 (α1c) antibody (Alamone Labs, Jerusalem, Israel). After washing three times with PBS, cells were incubated for 2 hrs. at room temperature with Alexa Fluor 633 (Molecular Probes, Oregon) goat anti-rabbit secondary antibody, followed by washing three times with PBS. The cells were incubated with 1 nM 17β-E2-6-(o-carboxymethyl)-oxime; (bovine serum albumin) BSA fluorescein isothiocyanate (FITC) and 1 nM (-) ST-BODIPY-DHP, (4,4-difluoro-7 steryl-4-bora-3a,4a-diaza)-3-(s-indacene) propionic acid, (Molecular Probes, Eugene, OR) for 15 mins. in the presence or absence of nonfluorescent (S)(-)-Bay K 8644 (Sigma, St Louis, MO). Unconjugated FITC and E2 were removed by microcon-10 centrifugation. The cells were washed twice with PBS, and imaged with Zeiss LSM 410 confocal microscope. Excitation of 633 nM and emission of 665 nM were used for Alexa Fluor 633, 480 nM excitation and 510 nM emission for FITC-conjugated E2, and 568 nM excitation and 590 nM emission for (-)
ST-BODIPY-DHP. For HEK 293 cell binding assay, cells were transfected with a 2:1:1 ratio of plasmid DNA comprised of either wild-type or mutant α1C (T1065Y), and β1b and α2δ L-type subunits by Lipofectamine 2000 reagent.

**Preparation of Membranes**

Transfected HEK 293 cells were washed, scraped, and homogenized using a glass teflon homogenizer in buffer X containing 50 mM Tris, 100 μM phenylmethylsulphonyl fluoride (PMSF), 100 μM benzamidine, 1 μM pepstatin A, 1 μg/mL leupeptin, and 2 μg/mL aprotinin, pH 8.0. The homogenate was centrifuged at 1000g for 5 mins. The supernatant was collected and centrifuged at 100,000g in a Beckman ultra centrifuge using a swinging (SW) 41 rotor for 1 hr. at 4°C. The membrane pellet was washed and resuspended in buffer X.

**Radioligand Binding**

Equilibrium binding assays were performed in buffer X containing 1 mM Ca²⁺ with 200 μg of membrane protein at 32°C for 3 hrs. Nonspecific binding was determined in the presence of 10 μM (±) PN200-110, and bound and free ligand were separated by vacuum filtration over glass fiber filters. Filters were washed with ice cold buffer containing 10 mM Tris, 1% polyethylene glycol 8000, 0.1% BSA, 0.01% Triton X -100, pH 8.0. Bound radioactivity was detected by liquid scintillation counting. For α1c competitive binding assay, various concentrations of cold E2 were used as the competing ligand. All the radioligand binding data was analyzed by GraphPad Prism analysis program version 5.01.
Construction and Properties of Mutant Calcium Channels

We used a mutant α1c subunit containing a threonine to tyrosine point mutation at position 1065 of rat brain coding sequence, which was constructed by Dr. Michael E. Greenberg, Harvard University (Dolmetsch et al., 2001). This mutant is homologous to rabbit heart α1c mutant, as shown in figure 6. Electrophysiological studies of rabbit heart α1c mutant (T1066Y, He et al., 1997) indicated that this mutant is insensitive to both agonist and antagonist without effecting the basal channel activity.

RESULTS

Estrogen potentiates L-type VGCC

Initial reports have shown that E2 induces a rapid rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{i}) in hippocampal neurons, but this induction is inhibited by an L-type calcium channel blocker (Wu et al., 2005). Whereas the authors indicated that estrogen-mediated activation of signaling events was responsible for this effect, an alternative possibility is that estrogen directly binds to and enhances the activity of the channel. To test this hypothesis, we measured the effects of estrogens on whole cell barium (Ba\(^{2+}\)) and Ca\(^{2+}\) currents in embryonic day 18 (E18) primary cultured rat hippocampal neurons. Ca\(^{2+}\) channel currents were isolated by inhibiting sodium (Na\(^{+}\)) currents with extracellular tetrodotoxin (TTX) and K\(^{+}\) channels with intracellular cesium (Cs\(^{+}\)) and extracellular 4-aminopyridine (4-AP), and either Ba\(^{2+}\) or Ca\(^{2+}\) was used as the charge carrier. The effect of E2 on the Ba\(^{2+}\) current-voltage (I-V) relationship was examined using brief (200 ms) step depolarization of 10 mV increments (from -80 to +50 mV) from a holding potential of -90 mV. The data revealed depolarization-activated
Ba$^{2+}$ currents ($I_{Ba^{2+}}$) recorded from a hippocampal neuron in the absence (control) or presence of 100 pM E2. The average amplitude of Ba$^{2+}$ current was 93 ± 13 pA in the control condition from a single hippocampal neuron. In the presence of 100 pM E2, the Ba$^{2+}$ currents were increased to 192 ±18% of the control (data not shown). To determine whether the effect of estrogens on Ca$^{2+}$ channels is concentration dependent, hippocampal neurons were exposed to various concentrations of E2. Mean peak $I_{Ba^{2+}}$ revealed a dose-dependent increase in Ba$^{2+}$ currents with as little as 10 pM E2 (data not shown). Effects of E2 were also very rapid. The onset of estrogen action was estimated to be less than 550 ms (data not shown).

Next, the goal was to determine which Ca$^{2+}$ channel subtype was being modulated by estrogens. About 1/3 of the elicited Ba$^{2+}$ current was due to activation of L-type VGCC, as evidenced by the inhibitory effects of the L-type VGCC inhibitor, nifedipine. Thus, it was assessed whether this channel may be a target of E2. Nifedipine (10 µM) nearly completely abolished the E2-induced potentiation of Ba$^{2+}$ current, indicating E2’s ability to enhance Ca$^{2+}$ current is due fully to potentiation of L-type VGCC (data not shown).

**Estrogen Potentiates Recombinant Ca$_{v1.2}$ VGCC in the Absence of Estrogen Receptors**

Of the L-type VGCC in the hippocampus, the predominate isoform is Ca$_{v1.2}$ (Hell et al., 1993; Davare et al., 2001; Sinnegger-Brauns et al., 2004). Synaptic and extrasynaptic localizations of Ca$_{v1.2}$ L-type VGCC correspond to putative roles of L-type calcium currents in synaptic modulation and in the propagation of dendritic Ca$^{2+}$ spikes.
To investigate the direct action of estrogen on L-type Ca\textsubscript{v}1.2, we assessed the action of E2 in HEK 293 cells transiently co-transfected with the pore forming subunit Ca\textsubscript{v}1.2, the accessory β1b and α2δ subunits, and GFP expression plasmids. HEK 293 cells do not endogenously express either L-type VGCC or ERs (Peterson et al., 1997; Thomas et al., 2005). Transfected HEK cells (GFP positive) showed that the expected Ca\textsuperscript{2+} current was activated in response to the same depolarizing protocol (Figure 1A and 1B). However, in the absence of transfection of the neuronal L-type Ca\textsuperscript{2+} channel, no current could be elicited in these cells in response to depolarization, thus providing a model to assess the dependence of the observed E2 response on these two entities. As we observed in neurons, exposure of recombinant L-type VGCC to E2 resulted in a significantly enhanced Ca\textsuperscript{2+} current (Figure 1C, 1D). These data support our contention that the E2 potentiated Ba\textsuperscript{2+}/Ca\textsuperscript{2+} current in hippocampal neurons is through the L-type Ca\textsubscript{v}1.2 Ca\textsuperscript{2+} channel via a mechanism independent of the ER.

**Estrogen-induced L-type VGCC Potentiation Mechanism**

The mechanism by which estrogens potentiate L-type VGCC was also addressed. One potential mechanism involves estrogen-induced rapid activation of L-type associated kinases such as PKA, PKC, and/or CaMKII. These kinases are known to facilitate L-type VGCC (Kamp and Hell, 2000; Hudmon et al., 2005). To assess this possibility, we used the pharmacological inhibitors of phospholipase C (PLC)/PKC and CaMKII. Our data revealed that neither PLC nor CaMKII inhibitors had an effect on estrogen-induced activation of L-type VGCC (data not shown).
Next, we explored the possibility that potentiation of L-type VGCC occurs as a result of direct binding of estrogen with the channel. It has been shown that estrogen and the estrogen-like compound, F90927, directly potentiate Maxi-K (Valverde et al., 1999) and L-type VGCC (Keller et al., 2006), respectively. Electrophysiological studies using charged DHPs demonstrate that the binding site is accessible exclusively from the outside of the plasma membrane (Shaw and Quatran, 1996; Strubing et al., 1993). We assessed whole cell binding of E2 to antagonist binding sites on L-type VGCC. As shown in Figure 2, an L-type VGCC antibody (Figure 2, red) and E2-FITC-BSA (Figure 2, green) showed membrane localization in HT-22 cells. This binding pattern resembled that of binding fluorescent (-) ST-BODIPY-DHP (Figure 2, blue), an antagonist for L-type VGCC (Keller et al., 2006). Additionally, treatment of cells with an excess of nonfluorescent Bay K (1 µM), an L-type VGCC agonist, reduced both E2-FITC-BSA and DHP binding, but did not affect L-type VGCC antibody binding (Figure 2), suggesting that E2 may bind to the same DHP agonist/antagonist binding region of L-type VGCC. We confirmed this idea by launching a two-pronged experimental approach. Competitive binding assay using membrane preparation from HEK 293 cells transiently transfected with wild-type α1c and the accessory subunits, showed that E2 competes with the radioligand [3H] PN200-110 for α1c binding with an IC50 of 0.67 nM (Figure 3). Because competitive binding is done on membranes that are electrically neutral, and electrophysiological effects are voltage and channel state dependent, it is critical to study the coupling between estrogen binding and potentiation of calcium current. Thus, we used a specific mutant of α1c channel which is insensitive to both DHP agonists and
antagonists, and tested the effects of E2 on potentiation of calcium current. As shown previously (He et al., 1997), mutant channels comprised of Q1070M and T1066Y amino acid residues in motif III S5 of rabbit heart α1c subunit of the L-type VGCC, are insensitive to DHP agonist and antagonist. Motif III S5 of rabbit heart α1c is 100% homologous with the rat brain α1c used in our experiment (Figure 4). We therefore chose to test the effects of E2 on the neuronal α1c mutant T1065Y channel. We confirmed that the T1065Y expressing channel was insensitive to nifedipine (data not shown). Interestingly, the ability of E2 to potentiate the T1065Y mutant channel was greatly attenuated (Figure 5Aa, 5Ab). Furthermore, whole cell binding assays of transiently expressed wild-type α1c channels in HEK 293 cells (Figure 6) show expression of α1c channels as evidenced by binding of a channel-specific antibody (Figure 6A), binding of E2-FITC-BSA (Figure 6B), and binding of fluorescently labeled DHP antagonist (Figure 6C). There was a markedly reduced binding of both E2-FITC-BSA (Figure 6F) and fluorescently labeled DHP antagonist (Figure 6G), but not an antibody that recognizes both wild-type and mutant α1c channel protein (Figure 6E), compared to wild-type channel. This effect, coupled with our findings that E2 competes with both a radiolabeled and a fluorescently-tagged DHP, suggest the binding domains overlap.
FIGURE 1

E2 potentiates recombinant neuronal L-type VGCC heterologously expressed in HEK 293 cells. HEK 293 cells were co-transfected with neuronal α1C, auxiliary subunits β1b and α2δ, and GFP with a DNA concentration of 10:1 (α1C, β1b, and α2δ:GFP). GFP expressing cells generally displayed robust neuronal L-type VGCC currents, while GFP negative cells had no detectable Ca\(^{2+}\) current (A). B) Fluorescent micrograph of GFP transfected HEK 293 cells. C) Representative whole cell current trace recorded from a GFP expressing cell in the presence or absence of 100 pM E2. D) Summary of E2 modulatory effect on whole cell Ca\(^{2+}\) current in HEK 293 cells transiently transfected with L-type VGCC. The current amplitudes were normalized to the control. *, p < 0.05, paired t-test, compared to the control. (All electrophysiology experiments were done by Dr. Ren-Qi Huang [Dr. Dillon’s Laboratory]).
Fluorescent negative cell
Fluorescent positive cell

10 ms
100 pA

Control

$17\beta$-E2

n=9

Percent control

Control

$17\beta$-E2

*
E2 as well as Bay K 8644 compete for the same fluorescent DHP binding site in HT-22 cells. Top panel shows confocal microscopy imaging from left to right: L-type VGCC α1c specific antibody staining of HT-22 cells (red), E2-FITC-BSA (green), (-) ST-BODIPY-DHP, (4,4-difluoro-7 steryl-4-bora-3a,4a-diaza)-3-(s-indacene) propionic acid, high affinity enantiomer (blue). Bottom panel shows that both fluorescent E2-FITC-BSA (1 nM) and fluorescent DHP (1 nM) binding was competed out in the presence of excess nonfluorescent DHP (Bay K 8644, 1 μM) as visualized by lesser fluorescent intensity compared to the control (top panel).
FIGURE 3

Competition binding curves for the displacement of 1 nM [3H] PN200-110 by varying concentrations of E2 in membranes from transiently transfected neuronal α1c, and the auxiliary subunits β1b and α2δ expression plasmids of L-type VGCC in HEK 293 cells. Insert shows typical equilibrium binding curves for [3H] PN200-110 alone.
FIGURE 4

Homology of rabbit heart and rat brain L-type VGCC α1c.
Homology of Rabbit heart and Rat brain L-type VGCC α1C

1066  1070
↓    ↓
NIVIVTLLLQFMCACIGVQLFKGK - Rabbit heart (GenBank accession No. 1509)

1065
↓
NIVIVTLLLQFMCACIGVQLFKGK - Rat brain (GenBank accession No.29789031)

As the T1066Y completely lost the sensitivity to both agonist and antagonist, we chose to test the effects of estrogen on mutant T1065Y.
FIGURE 5

A) Modulation of L-type VGCC by E2 in wild-type and mutant α1c channels transiently expressed in HEK 293 cells. Whole cell Ca\(^{2+}\) currents were recorded from the wild-type or mutant α1c T1065Y with a 55 ms depolarization pulse from a holding potential of -90 to 0 mV. E2 (100 pM) was applied in the bath for 3 mins. The ability of E2 to enhance Ca\(^{2+}\) currents was greatly attenuated in the DHP insensitive L-type VGCC. B) Mean results for these studies. The currents are normalized to the control (assigned as 100%). n = at least 4 cells. Note the sensitivity to E2 or nifedipine was significantly reduced in mutant channels (*, p < 0.05, unpaired t-test, compared to wild-type). (All electrophysiology experiments were done by Dr. Ren-Qi Huang [Dr. Dillon’s Laboratory]).
Mutant

10 ms

200 pA

Percentage stimulation

A

B

WT

n=9

Mutant

n=7

*
FIGURE 6

DHP and E2 binding characteristics of wild-type α1_c and mutant α1_c (T1065Y) L-type VGCC transiently expressed in HEK 293 cells. Left panels show confocal microscopy imaging of wild-type α1_c channels stained for (A) α1_c specific antibody (red), (B) E2-FITC-BSA (1 nM) binding (green), (C) (-) ST-BODIPY-DHP binding (blue), and (D) merge. Right panels show confocal microscopy imaging of mutant (T1065Y) α1_c channels stained for (E) α1_c specific antibody (red), (F) E2-FITC-BSA (1 nM) binding (green), (G) (-) ST-BODIPY-DHP binding (blue), and (H) merge.
DISCUSSION

The rapid interaction of estrogens with L-type VGCC and the resulting potentiation of voltage-induced Ca\(^{2+}\) currents could explain the observation that multiple and diverse signaling pathways are rapidly activated by estrogens. Calcium transients as a result of entry through L-type VGCC are known to activate Src kinase, GPCRs, MAPK/ERK, PI3K/Akt, PKA, and adenylyl cyclase signaling pathways (Morozov et al., 2003; Rusanescu et al., 1995; Dolmetsch et al., 2001; Chan et al., 2005; Vaillant et al., 1999). These rapid actions of estrogens appear to explain the observation that in pre-, peri-, and post-menopausal women, estrogens affect neuronal activity measured by functional magnetic resonance imaging (fMRI) in a variety of brain regions during the performance of cognitive (Dietrich et al., 2001), as well as sustained attentional tasks (Stevens et al., 2005). Further, estrogens enhance visual and place memory (Luine et al., 2003), working memory performances in rats (Daniel and Dohanich, 2001), and facilitate cholinergic neurotransmission in the septal-hippocampal pathways (Singh et al., 1994). Several mechanisms have been reported by which estrogen potentiates memory related synaptic plasticity not only slowly but also rapidly in the hippocampus. E2 has been shown to increase dendritic spinogenesis in the hippocampus (Woolley and McEwen, 1992; Chenjian et al., 2004), increase the expression of NMDAR subunit NR2B (Adams et al., 2004), and potentiate NMDAR mediated synaptic activity, including LTP (Woolley et al., 1997; Smith and McMahon, 2006). The identity of the estrogen receptor involved in
potentiation of synaptic plasticity and memory has not yet been fully confirmed. For example, in one report ERα but not ERβ (Ogiue-Ikeda et al., 2007), yet in another report ERβ but not ERα (Liu et al., 2008), regulates hippocampal synaptic plasticity and enhances cognitive ability. Our data suggest a new possible mechanism by which estrogen, via L-type VGCC potentiation, modulates memory related synaptic plasticity.

The lack of involvement of ERs in the observed potentiation by E2 of whole cell hippocampal Ca\(^{2+}\) currents is supported by two observations. First, the potentiation was seen at 10 pM E2, a concentration that is 500-fold lower than the EC50 of E2 for either ERα or ERβ. Secondly, HEK 293 cells transfected with the essential components of the L-type VGCC but lacking ERs (Peterson et al., 1997; Thomas et al., 2005) also responded potently to E2.

We also studied the mechanism underlying the estrogen modulation of VGCC. The lack of effect of CaMKII or PLC/PKC inhibitors does not support that the observed estrogenic action is initiated by these intracellular signaling pathways. However, the very rapid onset of E2 action, which was estimated to be less than a second, supports a direct interaction of E2 with the channel protein. We confirmed this idea by 1) whole cell binding assay using fluorescent ligand in a hippocampal derived neuronal cell line, HT-22 cells, where α1c channel is endogenously expressed, 2) whole cell binding in HEK 293 cells transiently expressed with mutant and wild-type α1c channel, 3) competitive binding assay using radioligand, and 4) electrophysiological studies using wild-type and DHP insensitive channels. The displacement of a L-type VGCC agonist, Bay K 8644, by E2 also provides evidence for this direct mechanism. Furthermore, it is worthy to note
that a structurally similar estrogen-like compound, F90927, has recently been shown to directly modulate L-type VGCC in myocytes (Keller et al., 2006). Our studies indicate that estrogen itself binds with high affinity to the L-type VGCC, at a domain that overlaps with the DHP site.

Functional consequences of estrogen-induced potentiation of L-type VGCC in hippocampal neurons are now becoming clear. Neuronal activity-dependent potentiation of L-type VGCC has an important role in synaptic plasticity and in memory (Magee and Johnston, 1997; Sjostrom and Nelson, 2002; Bading et al., 1993). Recently, the function of L-type VGCC in spatial learning, synaptic plasticity, and triggering of learning associated biochemical processes, were evaluated in a transgenic mouse with an inactivation of Cav1.2 gene in the hippocampus and neocortex (Moosmang et al., 2005). This study showed selective loss of protein synthesis-dependent but NMDAR-independent LTP, a severe impairment of hippocampus-dependent spatial memory, loss of activation of MAPK/ERK pathway, and repressed cAMP response element-dependent transcription in hippocampal neurons. Also very recently it has been shown that the activity of L-type VGCC is important for spike-timing-dependent LTP, which is absent in Fragile X syndrome (Meredith et al., 2007). Therefore, we speculate that estrogen-induced, direct potentiation of L-type VGCC could have implications in modulating synaptic plasticity and memory formation.
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ESTROGEN RAPIDLY INDUCES PHOSPHORYLATION OF PLASTICITY RELATED KINASES AND ATTENUATES INHIBITION OF GLUR1 PHOSPHORYLATION BY AMYLOID-BETA OLIGOMERS

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CHAPTER III. ESTROGEN RAPIDLY INDUCES PHOSPHORYLATION OF PLASTICITY RELATED KINASES AND ATTENUATES INHIBITION OF GLUR1 PHOSPHORYLATION BY AMYLOID-BETA OLIGOMERS

ABSTRACT

Neuromodulation of synaptic plasticity by 17β-estradiol (E2) is thought to influence information processing and storage in the cortex and hippocampus. Because E2 rapidly affects cortical memory and synaptic plasticity we examined its effects on phosphorylation of CaMKII, ERK, and AMPAR (GluR1 subunit), all of which are important for the induction and maintenance of synaptic plasticity and memory. As these phosphorylation events mechanistically link increased dendritic spine growth and spine density, we also examined estrogen-induced alterations in dendritic spine shape and spine density in cortical neurons. Because soluble amyloid-beta (Aβ) oligomers inhibit synaptic plasticity, we also tested E2’s potency for ameliorating Aβ-induced dysfunction of synaptic plasticity. Acute E2 treatment resulted in an increased temporal and spatial phosphorylation pattern of CaMKII, ERK, and AMPAR (GluR1 subunit). We then found that E2 rapidly increased excitatory synapse spine density. Finally, we found that E2 ameliorated Aβ-induced inhibition of pGluR1 and decreased mushroom-type spine density. This study identifies E2-induced signaling pathways that link structural and functional synaptic plasticity in
cortical neurons, which may facilitate functional recovery from Aβ-induced synapse dysfunction.

**The abbreviations used are:** E2, 17β-estradiol; LTP, long-term potentiation; LTD, long-term depression; AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GluR1, AMPA-type glutamate receptor subunit 1; NMDAR, N-methyl-D-aspartate receptor; L-type VGCC, L-type voltage-gated calcium channels; Aβ, amyloid-beta; CaMKII, calcium/calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinase; AD, Alzheimer’s disease.
INTRODUCTION

Activity-dependent rapid structural and functional modulations of central excitatory synapses contribute to synapse formation, experience-dependent plasticity, learning, and memory. In forebrain pyramidal neurons, induction of long-term potentiation (LTP) causes rapid enlargement of spine heads and simultaneous delivery of AMPAR (GluR1 subunits) into spines (Engert and Bonhoeffer, 1999; Kopec et al., 2006; Matsuzaki et al., 2004). Similar modifications have been observed in the cortex in response to LTP (Connor et al., 2006), or experience (Takahashi et al., 2003). It is now well established that NMDAR and L-type VGCC-dependent activation of CaMKII is necessary for both structural and functional synaptic plasticity (Matsuzaki et al., 2004; Lee et al., 2009). CaMKII, which is highly abundant in spines, is activated and autophosphorylated during LTP (Lisman et al., 2002). In the primary somatosensory cortex of α-CaMKII knock-out mice or knock-in mice with a form of CaMKII that can not be phosphorylated at threonine 286, failed to show cortical plasticity and cortical LTP (Glazewski et al., 1996; Glazewski et al., 2000; Frankland et al., 2001). Experiments in the visual cortex show similar effects of the T286A mutation on an experience-dependent plasticity (Taha et al., 2002).
Plasticity at synapses can be regulated at presynaptic sites by changing the release of neurotransmitter molecules, or postsynaptically by changing the number, types, or properties of neurotransmitter receptors. Studies using in vitro synaptic plasticity models have identified the regulated trafficking of postsynaptic AMPAR type glutamate receptors a crucial mechanism underlying activity-induced changes in synaptic transmission (Newpher and Ehlers, 2008). Excitatory synapses contain AMPAR to transmit signals and NMDAR to trigger long-term changes in synaptic transmission (LTP and long-term depression [LTD]). While many mechanisms involve regulating the onset or magnitude of LTP and LTD, in many cases there appears to be one common mechanism, that is the addition and removal respectively of synaptic AMPAR (Bredt and Nicoll, 2003; Malinow and Malenka, 2002). The mechanisms by which GluR1 containing receptors are driven to synapses during LTP is not fully elucidated. However, it has been shown that several activity driven phosphorylation events at the C-terminal of GluR1 by PKA at serine 845 (Roche et al., 1996), by CaMKII and PKC at serine 831 (Boehm et al., 2006) facilitate synaptic AMPAR delivery (Esteban et al., 2003; Song and Huganir, 2002). Using phosphospecific antibodies AMPAR phosphorylation states can be monitored in neurons to assess changes in potentiation of synaptic transmission (Lee et al., 2000). Therefore, manipulating and monitoring phosphorylation and AMPAR trafficking provides an effective means to study cognitive function and dysfunction in animal models.
LTP-inducing stimuli causes the formation of new spines (Matsuzaki et al., 2004; Nagerl et al., 2004; Okamoto et al., 2004), whereas LTD-inducing stimulation is associated with shrinkage and or retraction of spines (Zhou et al., 2004). Soluble Aβ oligomers decrease cell surface expression of NMDAR and AMPAR, inhibits LTP, inhibits phosphorylation of CaMKII, ERK, and GluR1, and decreases spine density (Snyder et al., 2005; Shrestha et al., 2006). Furthermore, Aβ dimmers isolated directly from cerebral cortex of subjects with Alzheimer’s disease (AD), potently inhibited LTP and reduced dendritic spine density (Shankar et al., 2008). In the context of the decreased synaptic density observed in the hippocampus and neocortex of AD patients, these findings suggest that soluble Aβ can perturb synaptic plasticity and lead to spine loss. Spine loss was prevented by Aβ specific antibody or a small molecule modulator of Aβ aggregation, scyllo-inositol (AZD 103) [Shankar et al., 2007]. In an effort to understand whether E2-induced biochemical signaling mechanistically links synaptic plasticity, we have determined the phosphorylation patterns of CaMKII, ERK, and AMPAR (GluR1 subunit), and the spine structure analysis, in vitro as well as in vivo in cortical neurons.
MATERIALS AND METHODS

Primary Neuronal Culture

At embryonic day 18 (E18), pregnant rats were anesthetized and cervically dislocated. The brains of pups were removed and placed into magnesium (Mg\(^{2+}\)) free Hank’s balance salt solution (HBSS). Cortices were removed under a dissecting microscope, washed, and placed into neurobasal culture media (without phenol red) supplemented with B27 and pen-strep (all from Gibco, Carlsbad, CA). The cortices were triturated using a graded series of fine polished Pasteur pipettes, and then filtered through a 40 µm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). The neurons were plated on poly-L-lysine coated 100 mm dishes and glass coverslips, and cultured in vitro in 95% humidity and 5% CO\(_2\) atmosphere. At day 2 cells were treated with 5 µM 1-beta-d-arabinofuranosylcytosine (AraC) to inhibit glial cell growth.

Western Blot Analysis

To measure estrogen-induced changes in protein phosphorylation, 3 samples for each experimental treatment were used for western blot analysis. After the respective treatments, cortical neurons were lysed with 100 µL of ice cold buffer containing 50 mM Tris, 10 mM Mg\(^{2+}\), 1 mM EDTA, 1 mM EGTA, 10 mM benzamide, 100 ng/ml leupeptin, 100 ng/ml aprotinin, 0.08 mM sodium molybdate, 0.01% tritonX-100, 10 µM okadoic acid, and 2 mM sodium pyrophosphate, pH 7.4. Aliquots of the lysed
and sonicated homogenate were taken to determine protein concentration using protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Samples containing 30 µg protein were electrophoresed on a SDS/PAGE gel. The protein was transferred onto PVDF membrane (Millipore, Billerica, MA), blocked for 1 hour with PBS containing 5% non-fat dried milk, and probed overnight at 4°C with primary antibody. A polyclonal antibody against the alpha subunit of CaMKII phosphorylated at threonine 286 (Cell Signaling Technology, Danvers, MA) was used at a dilution of 1:1000 to detect activation of the kinase (autophosphorylation). A monoclonal antibody against ERK phosphorylated at tyrosine 204 (Santa Cruz, CA) was used at a dilution of 1:1000 to detect activation. A polyclonal antibody was used at a dilution of 1:1000 to recognize GluR1 phosphorylation at the CaMKII site (serine 831, Upstate, Temecula, CA). A polyclonal antibody was used at a dilution of 1:1000 to recognize GluR1 phosphorylation at the PKA site (serine 845, Upstate, Temecula, CA). After washing 3 times with PBS, the membranes were further incubated at room temperature with horseradish peroxidase conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA) at a dilution of 1:1000. The proteins were visualized with supersignal chemiluminescence (Pierce Biotechnology, Rockford, IL) using UVP software (Upland, CA). For the loading control, membranes were stripped and reprobed with total antibody. Antibodies for total CaMKII, total ERK, total GluR1, and gamma-aminobutyric acid (GABA) were used at a dilution of 1:1000.
Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes. After a 1 hour block with 5% bovine serum albumin (BSA), coverslips were incubated with the appropriate primary antibody (anti-phospho-CaMKII; anti-phospho-ERK1/2) over night at 4°C, then washed with PBS (three 5 minute washes) and incubated with the appropriate secondary antibody (goat anti-mouse or goat anti-rabbit Alexa Fluor 633, Invitrogen, Carlsbad, California) for 1 hour, followed by 3 PBS washes. Samples were visualized using laser scanning confocal microscopy.

Live Animal Drug Administration and Tissue Procurement

Female Charles River Sprague-Dawley rats were maintained in laboratory acclimatization for 3 days before ovariectomy. All animal procedures were reviewed and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Community. Ovariectomized rats were used to study the in vivo effects of E2. Bilateral ovariectomy was performed 3 weeks prior to subcutaneous drug administration. E2 was dissolved in absolute ethanol and then corn oil (Penta Manufacturing, Airfield, NJ) at a concentration of 320 µg/ml. Ethanol was evaporated by incubation at 50°C overnight. A single subcutaneous injection of 300 µl E2 (320 µg/kg) or vehicle was administered, as described by our laboratory (Yang et al., 2003).

Soluble Aβ Oligomer Preparation

Synthetic Aβ1-42 (Tocris, Ellisville, Missouri) was prepared without the fibrillar component according to the protocol described by Lambert et al., 2001. In brief, Aβ1-
42 was dissolved in anhydrous dimethyl sulfoxide (DMSO) to 5 mM, which was then added to ice cold neurobasal medium without phenol-red to 100 µM. This solution was incubated at 4°C for 24 hours, and then centrifuged at 14,000g for 10 minutes. The supernatant comprised of fibrillary-free oligomers, as well as, monomers, was used for the experiments performed in this study. Protein concentration was determined from the supernatant and its molarity was calculated (0.5 µM).

**Surface Labeling**

Cortical neurons, while still in the petry dish, were incubated with PBS containing 1.5 mg/ml EZ-link sulfo-NAS-LC-biotin (Pierce Biotechnology, Inc., Rockford, IL) for 20-30 minutes at 4°C. Cells were removed from the dish, washed with PBS, and then centrifuged. Washed cells were lysed and the supernatant was incubated with immobilized neutravidin protein (Pierce Biotechnology, Inc., Rockford, IL) for 1 hour at room temperature. Protein beads were obtained by centrifugation and washed 3 times with PBS. Protein beads were dissolved in sodium dodecyl sulfate (SDS) sample buffer.

**Statistical Analysis**

Densitometric analysis of western blots was conducted using LabWorks Image Acquisition and Analysis software (UVP, Inc., Upland, CA). Densitometric data from at least three independent experiments was subjected to ANOVA, followed by Newman-Keuls Multiple Comparison Test for the assessment of group differences, and was presented as a bar graph depicting the average ± SD, using GraphPad Prism software (La Jolla, CA).
Production of Recombinant Adeno-Associated Virus 2 (AAV2) and Infection of Cortical Neurons

The helper plasmid pDG, which contains both the AAV2 genes (rep and cap) and helper genes, (Grimm et al., 1998) was used to generate recombinant AAV2. Recombinant AAV2 vectors were packaged, purified, concentrated, and titered by the following method: Zolotukhin et al., 1999. Recombinant GFP containing AAV2 particles were used at a multiplicity of infection (MOI) of 100. 3-4 days after infection the respective treatments were added. Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes. Samples were visualized with laser scanning confocal microscopy.

Dendritic Spine Density and Spine Structure Analysis

GFP-expressed cortical neurons were used for treatment with soluble Aβ oligomers (0.05 µM) for 24 hours. Spine structure alteration was studied in Aβ oligomer treated, untreated, and in the presence or absence of E2 (10 nM) for 1 hour. For the spine structure and spine density analysis the cortical neurons expressing GFP were used for imaging. Cortical neurons were imaged by one-photon laser scanning microscopy using Zeiss LSM510. Images of individual cortical pyramidal-type GFP-expressing neurons were used to make filament objects, spines, and dendrites with Imaris XT software (Bitplane, Saint paul, MN). From the traces, stubby, mushroom and thin type spines were sorted using MatLab XT software (Bitplane, Saint paul, MN). Finally, spines were classified by using “classify spines” program from the MatLab
software and this was displayed as color coded spine heads, for example red, blue, and green as thin, mushroom, and stubby respectively.

RESULTS

Estrogen Rapidly Increased Phosphorylation of CaMKII, ERK, and AMPAR (GluR1 subunit) in Primary Cortical Neurons

Estrogen has been shown to exert acute affects on synaptic physiology involving synaptic plasticity related kinases such as CaMKII (Sawai et al., 2002) and ERK in primary hippocampal neurons (Lee et al., 2004). Little is known about the temporal regulation of these kinases in cortical neurons. Therefore, we decided to investigate the phosphorylation state of these kinases at different time points. E2 (10 nM) treatment in primary cortical neurons resulted in a steady increase of normalized pCaMKII immunoreactivity from basal, starting 5 min. after E2 treatment and with its maximum at 20 min. (592 ± 183%, compared to control, n=3, p<0.001) [Figure 1A.], followed by a decline in phosphorylation at 30 min. (427 ± 27%, compared to control, n=3, p<0.01) and 60 min. (324 ± 50%, compared to control, n=3, p<0.05) [Figure 1A.]. Next we studied ERK activity by measuring its phosphorylation state. E2 (10 nM) treatment also resulted in a steady increase of normalized pERK immunoreactivity from basal, starting 5 min. after E2 treatment and with its maximum at 30 min. (1827 ± 73%, compared to control, n=3, p<0.001) [Figure 1B.], followed by a decline in phosphorylation at 60 min. (1074 ± 204%, compared to control, n=3, p<0.001). We next sought to determine whether E2-induced activation of CaMKII can increase phosphorylation of its substrate GluR1 (CaMKII site serine
Using serine 831 pGluR1 antibody we showed that E2 treatment in primary cortical neurons lead to the increase of GluR1 phosphorylation starting at 30 min. (204 ± 20%, compared to control, n=3, p<0.05) and phosphorylation was maximal at 60 min. (403 ± 88%, compared to control, n=3, p<0.001) [Figure 1C.]. To further investigate the phosphorylation state of CaMKII and ERK, we determined the spatial distribution of these kinases in primary cortical neurons. Immunofluorescent micrograph showed that E2 treatment for 20 and 30 min. increased phosphorylation of CaMKII and ERK respectively. This increased phosphorylation was visible in both the cell body and membrane of the dendrites (Figure 1D. and 1E.).

**Acute Estrogen Treatment Increased Phosphorylation of CaMKII, ERK, and AMPAR (GluR1 subunit) in Cortical Neurons In vivo**

We next asked whether acute E2 treatment would have a similar effect on the phosphorylation state of CaMKII, ERK, and GluR1 in vivo as observed in vitro. For the in vivo studies, E2 was introduced subcutaneously (320 µg/kg) in ovariectomized rats and the phosphorylation state of CaMKII, ERK, and GluR1 was determined at distinct periods (3, 6, and 24 hrs.). The results for normalized pCaMKII identified an increase from basal level at 3 hrs. (152 ± 19%), with its maximum at 6 hrs. (227 ± 37%, compared to control, n=3, p<0.01), followed by rapid return to basal level (143 ± 46%) [Figure 2A.]. For pERK, phosphorylation was highest at 6 hrs. (316 ± 36%, compared to control, n=3, p<0.001), followed by a return to basal level at 24 hrs. (150 ± 20%) [Figure 2B.]. In the case of GluR1, phosphorylation was maximum at 6 hrs.
(353 ± 116%, compared to control, n=3, p<0.01) and returned to basal level at 24 hrs. (85 ± 28%) [Figure 2C.].

The CaMKII Inhibitor, KN-93, Inhibited Phosphorylation levels of GluR1 in Primary Cortical Neurons

To determine which of the activated kinases (CaMKII, ERK, and/or PI3K) are necessary for the increased phosphorylation of GluR1, we used primary cortical neuronal culture and treated with CaMKII inhibitor KN-93, MEK inhibitor U0126, and PI3K inhibitor LY29402. Treatment with KN-93 for 30 min. resulted in no inhibition of phosphorylation of GluR1 (E2, 140 ± 6%, compared to E2 and KN-93, 120 ± 16%), whereas, KN-93 treatment for 60 min. severely inhibited phosphorylation of GluR1 at its CaMKII site (serine 831) [E2, 431 ± 24%, compared to E2 and KN-93, 169 ± 19%, n=3, p<0.001] (Figure 3). MEK inhibitor, U0126, had no inhibitory effect on the phosphorylation of GluR1 as shown in Figure 3 (E2, 431 ± 24%, compared to E2 and U0126, 442 ± 40%). PI3K inhibitor, LY29402, was similar to that of U0126, in that it too had no inhibitory effect on the phosphorylation of GluR1 (Figure 3) [E2, 431 ± 24%, compared to E2 and LY29402, 401 ± 40%].

Soluble Aβ1-42 oligomers Abrogated and E2 Ameliorated Phosphorylation of GluR1 at its CaMKII Site in Primary Cortical Neurons

It has been reported that soluble Aβ1-42 treatment in hippocampal neurons, inhibits phosphorylation of both CaMKII and GluR1 (serine 831 site) [Zhao et al., 2004]. To determine the effect of Aβ in cortical neurons, we analyzed the phosphorylation state...
of GluR1 at its CaMKII site. As shown in figure 4, soluble Aβ₁-₄₂ (0.5 µM) oligomer exposure for 24 hrs. decreased GluR1 phosphorylation (63 ± 10%), while E2 (10 nM) treatment increased phosphorylation of GluR1 (455 ± 152%, compared to control, n=3, p<0.001). E2 (10 nM) exposure for 1 hr. to Aβ (0.5 µM, 24 hrs.) pretreated primary cortical neurons and in the presence of Aβ, ameliorated Aβ-induced inhibition of phosphorylation of GluR1 (E2 and Aβ, 317 ± 38%, compared to Aβ, 63 ± 10%, n=3, p<0.01) [Figure 4.]. The figure also shows that E2-induced phosphorylation of GluR1 was inhibited by CaMKII inhibitor (KN-93) [E2, 455 ± 152%, compared to E2 and KN-93, 218 ± 55%, n=3, p<0.01]. KN-93 treatment alone inhibited the basal phosphorylation level of GluR1 (KN-93, 59 ± 20%).

**E2 Prevented and Soluble Aβ₁-₄₂ Inhibited GluR1 Trafficking in Primary Cortical Neurons**

It has been previously documented that activated CaMKII can modulate synaptic plasticity by enhancing AMPAR channel conductance via GluR1 phosphorylation at its CaMKII site (Barria et al., 1997) and by delivering AMPAR to the synapse (Poncer et al., 2002). Thus we sought to determine whether E2 treatment prevents Aβ₁-₄₂ inhibition of GluR1 insertion into the surface membrane. We used the surface labeling technique of biotinylation in primary cortical neurons to quantify GluR1 surface expression. As shown in figure 5, E2 (10 nM, 1 hr.) treatment increased surface expression of GluR1 (338 ± 46%, compared to control, n=3, p<0.001), whereas, 24 hr. soluble Aβ₁-₄₂ oligomer treatment inhibited GluR1 insertion into the
membrane of cortical neurons (49 ± 10%). Aβ-induced inhibition of GluR1 insertion was ameliorated by E2 treatment (E2 and Aβ, 270 ± 17%, compared to Aβ, 49 ± 10%, n=3, p<0.001) [Figure 5].

**Estrogen-induced Signaling Rapidly Increased Spine Number and Ameliorated Aβ1-42-induced Spine Loss in Primary Cortical Neurons**

Studies in animal models have demonstrated that acute (less than 1 hour) systemic E2 injections result in an improvement of cognitive performances that rely on hippocampal and cortical information processing (Li et al., 2004; Sinopoli et al., 2006). Improvement of these performances are thought to underlie E2-mediated changes in synaptic function and dendritic spine structure (Mukai et al., 2007; Woolley, 2007). However, the signaling pathway(s) that regulate E2-mediated changes in dendritic spine structure and spinogenesis have not been investigated. To examine whether E2 can rapidly change synaptic architecture we analyzed dendritic spine morphology in matured cultured cortical neurons infected with AAV2-GFP virus. GFP-expressing cortical neurons were treated with 10 nM E2 for a maximum of 2 hrs. E2 treatment increased the number of specific classes of spines which included the excitatory synapse specific mushroom-type spine (0.59 ± 0.02 spines/µm, compared to control, 0.30 ± 0.02 spines/µm, n=3, p<0.001, Figure 7). Also, long-thin-type spine number increased as a result of E2 treatment (0.67 ± 0.04 spines/µm, compared to control, 0.39 ± 0.02 spines/µm, n=3, p<0.001, Figure 7). E2-induced increase in mushroom-type spine density was abrogated by CaMKII inhibitor
(KN-93) [E2 and KN-93, 0.20 ± 0.02 spines/µm, compared to E2, 0.59 ± 0.02 spines/µm, n=3, p<0.001), whereas, MEK inhibitor (U0126) had no effect (Figure 7). We next asked whether E2 can exert ameliorating effects on cortical neurons that had been exposed to soluble Aβ oligomers. In the context of loss of synaptic density observed in the hippocampus and neocortex of patients with AD, it has been suggested that the soluble form of Aβ can lead to synapse loss (Shankar et al., 2008). To determine whether soluble Aβ triggers synapse loss, we studied the effect of Aβ oligomers in mature cortical neurons. 24 hr. treatment with soluble Aβ oligomers (0.5 µM) severely reduced spine density (specifically mushroom-type) (0.19 ± 0.02 spines/µm, compared to control, 0.30 ± 0.02 spines/µm, n=3, p<0.001) [Figure 7]. E2 attenuated Aβ-induced loss of spine density (E2 and Aβ, 0.39 ± 0.02 spines/µm, compared to Aβ, 0.19 ± 0.02 spines/µm, n=3, p<0.001) [Figure 7].
FIGURE 1

Estrogen induces phosphorylation of CaMKII, ERK, and GluR1 in primary cortical neurons. E2 (10 nM) temporally regulated the phosphorylation of CaMKII, ERK, and GluR1. A.) Phosphorylation is seen within 5 min. and is maximum at 20 min. for CaMKII (592 ± 183%), while B.) ERK phosphorylation is seen within 5 min. but is maximum at 30 min. (1827 ± 73%). C.) GluR1 is phosphorylated at 30 min. but is maximal at 60 min. (403 ± 88%). E2 also induces phosphorylation of CaMKII and ERK in the cell body and dendritic extensions of primary cortical neurons. Cortical neurons untreated or treated with E2 (10 nM) for either 20 min. or 30 min. were labeled with D.) phospho-CaMKII or E.) phospho-ERK respectively and visualized by fluorescence microscopy. Data are ± SD. *, p<0.05, **, p<0.01, ***, p<0.001, one-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=3 (3 independent experiments).
A.

pCaMKII

CaMKII

Percent of Control

0 min.  5 min.  10 min.  20 min.  30 min.  60 min.

E2 (10 nM)
D. pCaMKII

Control  E2
FIGURE 2

Acute E2 treatment increases phosphorylation of CaMKII, ERK, and GluR1 in cortical neurons in vivo. Subcutaneous injection (320 µg/kg) of E2 in ovariectomized rats significantly increased phosphorylation of A) CaMKII, B) ERK, and C) GluR1 6 hrs. after injection (227 ± 37%, 316 ± 36%, and 353 ± 116%, respectively), and all rapidly returned to their respective basal levels at 24 hrs. Bar graphs are relative density of A) pCaMKII, B) pERK, and C) pGluR1 bands compared to control (assigned as 100%). Data are ± SD. *, p<0.05, **, p<0.01, ***, p<0.001, one-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=3 (3 independent experiments).
FIGURE 3

Estrogen-induced phosphorylation of GluR1 was inhibited by CaMKII inhibitor, KN-93, in primary cortical neurons. Primary cortical neurons (E18) were grown 15 days in vitro (DIV) and treated with E2 (10 nM) in the presence or absence of CaMKII inhibitor (KN-93, 5 µM), MEK inhibitor (U0126, 10 µM), and PI3K inhibitor (LY29402, 10 µM) for 30 and 60 min. Induction of phosphorylation of GluR1 at serine 831 was inhibited by KN-93 (169 ± 19%) but not U0126 (442 ± 40%) and LY29402 (401 ± 40%) compared to 60 min. vehicle-treated (DMSO) control. Data are mean ± SD. **, p<0.01, ***, p<0.001, two-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=3 (3 independent experiments).
FIGURE 4

E2 ameliorates Aβ-induced inhibition of GluR1 phosphorylation in primary cortical neurons. Primary cortical neurons (E18) were grown 15 DIV and were pretreated with soluble Aβ_{1-42} oligomers (0.5 µM) for 24 hrs. Neurons were then treated with or without E2 (10 nM) for 1 hr. in the presence or absence of KN-93. Aβ treatment decreased GluR1 phosphorylation (63 ± 10%), while E2 ameliorated Aβ’s effect (317 ± 38%). E2-induced phosphorylation was inhibited by KN-93 (218 ± 55%). Data are mean ± SD. **, p<0.01, ***, p<0.001, one-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=3 (3 independent experiments).
FIGURE 5

GluR1 insertion into the membrane of primary cortical neurons. Primary cortical neurons (E18) were grown 15 DIV and were pretreated with soluble Aβ_{1-42} oligomers (0.5 µM) for 24 hrs. E2 (10 nM) treatment for 1 hr. increased surface expression of pGluR1 (serine 831) and Aβ treatment inhibited this surface expression (338 ± 46% and 49 ± 10%, respectively). The surface expression of pGluR1 (serine 831) was normalized with surface expression of GABA receptor. E2 treatment ameliorated Aβ-induced inhibition of GluR1 insertion into the membrane of cortical neurons (270 ± 17%). Data are mean ± SD. **, p<0.01, ***, p<0.001, one-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=3 (3 independent experiments).
FIGURE 6

Soluble Aβ_{1-42} oligomers decrease the density of mushroom-type spines and E2 ameliorates Aβ-induced spine loss in primary cortical neurons. Primary cortical neurons (E18) were grown 13 DIV then infected with AAV2-GFP viruses and further grown for 2 days. GFP-expressing cortical neurons were treated in the presence or absence of E2 (10 nM) for 2 hrs. (B. D. E. and F.), in the presence or absence of 24 hr. pretreatment with soluble Aβ_{1-42} oligomers (0.5 μM) [C. and D.], in the presence or absence of KN-93 (5 μM) for 2 hrs. (E.), and in the presence or absence of UO126 (10 μM) for 2 hrs. (F.). Immediately after treatments, neurons were fixed and one-photon confocal images were acquired. Representative examples of confocal images expressing GFP are shown (A., B., C., D., E., and F.-top panel). GFP images were then used for spine density analysis. Using Imaris XT software, first spines were assigned by filament tracing and then classified as stubby (red), mushroom (green), and long-thin (blue), using Imaris XT-MatLab spine classifier. Micrographs showing assigned spines in the whole neuron, cropped distal dendrites showing assigned spines, and respective spine counts shown in bar graphs are all depicted.
A. Control

![Image of neuron branches with spine counts chart]

- Stubby
- Mushroom
- Long Thin

Spine Counts
C. $\text{A}\beta$

![Image](image_url)

![Image](image_url)

![Image](image_url)

![Bar Chart](image_url)
E. E2 + KN-93

Spine Counts

![Neuron images with spine counts graph]

<table>
<thead>
<tr>
<th>Spine Type</th>
<th>Count</th>
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<tr>
<td>Stubby</td>
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</tr>
<tr>
<td>Mushroom</td>
<td>100</td>
</tr>
<tr>
<td>Long Thin</td>
<td>500</td>
</tr>
</tbody>
</table>
F. E2 + U0126

[Image of neural network diagrams with bar graph showing spine counts for different categories: S脱y, Mushroom, Long Tho]
FIGURE 7

Bar graph showing quantitative analysis of spine counts/µm dendritic length. E2 (10 nM) increased mushroom- and long-thin-type spine density (green) [0.59 ± 0.02 spines/µm and 0.67 ± 0.04 spines/µm, respectively]. Soluble Aβ1-42 oligomer (0.5 µM) treatment inhibited mushroom-type spine density (0.19 ± 0.02 spines/µm) without affecting other spine densities (stubby and long-thin, red and blue). E2 (10 nM) treatment ameliorates Aβ-induced mushroom-type spine density loss (0.39 ± 0.02 spines/µm). E2-induced increase in mushroom-type spine density was inhibited by CaMKII inhibitor KN-93 (0.20 ± 0.02 spines/µm), but not MEK inhibitor U0126. Data are mean ± SD. ***, p<0.001, two-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=6 (3 independent experiments).
DISCUSSION

Mounting evidence suggests that neuromodulation of synaptic plasticity is necessary for information processing and storage in hippocampal as well as cortical networks (Marder and Thirumalai, 2002). Recently estrogen’s neuromodulatory role has been documented by showing that E2 treatment enhanced glutamate release via rapid nongenomic action of PI3K in hypothalamic presynaptic neurons and enhanced dendritic spine formation (Schwarz et al., 2008). Moreover, the neuromodulatory role of E2 is thought to occur through local estrogen formation in the pyramidal cells of the hippocampus and neocortex, thus affecting the functions of excitatory synapses (Yague et al., 2008). E2-induced enhancement of structural and functional synaptic plasticity is evidenced by increased phosphorylation of plasticity related kinases, increased spine density, neuronal network connectivity, and synaptic transmission (Woolley, 2007; Spencer et al., 2008). E2-induced increases in structural and functional plasticity are associated with learning and memory, whereas, decreases are associated with cognitive dysfunction. Structural and functional synaptic plasticity dysfunction have been implicated as the neuropathological correlates of severity of cognitive dysfunction in AD. The signaling mechanism by which E2 modulates synaptic plasticity at the postsynaptic cortical neuron has not been well studied. We therefore investigated E2-induced signaling pathways and asked which signaling mechanism is responsible for linking structural and functional synaptic plasticity. We
also asked whether soluble Aβ-induced dysfunction of signaling pathways involved in synaptic plasticity can be attenuated by E2 treatment in cortical neurons.

We used here a cultured cortical neuronal system to elucidate the molecular components involved in E2-induced rapid signaling pathways which link structural and functional plasticity. We identified that activation of CaMKII is the signaling mechanism for the E2-mediated increase in GluR1 phosphorylation, as well as, spine density. Reduction in CaMKII activity by CaMKII inhibitor, KN-93, resulted in a decrease in the phosphorylation of GluR1 and excitatory synapse specific spine number. Previous studies have shown that E2-modulates LTP (Cordoba Montoya and Carrer, 1997; Foy et al., 1999) in the hippocampal region and increases CaMKII activation (Sawai et al., 2002). It is also well known that NMDAR and L-type VGCC activation of CaMKII is necessary for both structural (spine growth and spine number) and functional (induction and/or expression of LTP) plasticity in hippocampal neurons. Previously we have shown that estrogen directly potentiates L-type VGCC (Sarkar et al., 2008). Therefore, E2 by potentiating L-type VGCC in cortical neurons may activate CaMKII which in turn phosphorylates GluR1 and modulates LTP induction and/or expression. LTP-inducing stimuli causes the formation of new spines and enlargement of existing spines (Matsuzaki et al., 2004; Nagerl et al., 2004; Okamoto et al., 2004). Insertion of more AMPAR (containing GluR1 subunits) to activated synapses is known to be critical for LTP induction and/or maintenance (Malinow and Malenka, 2002) Phosphorylation of GluR1 by CaMKII also modulates synaptic plasticity by delivering AMPAR to the synapse
(Poncer et al., 2002). Our surface biotinylation studies revealed that E2 increased surface levels of GluR1 phosphorylated at serine 831. These findings are consistent with studies that serine 831 phosphorylation is critical for regulating subcellular trafficking of GluR1 (Liao et al., 2001). Also, our results raise the intriguing possibility that AMPAR can be modified and recruited rapidly to silent synapses via the E2-induced signaling mechanism in spontaneously activated primary cortical neurons. E2 has been reported to rapidly modulate LTP, LTD, spinogenesis, and promote new excitatory synapse specific spines (mushroom) in the dendrites of hippocampal neurons (Ogiue-Ikeda et al., 2008; Woolley et al., 1992; Li et al., 2004). In this study we observed that E2 treatment resulted in increased mushroom-type spines in the dendrites of cortical neurons without significantly affecting stubby- and long-thin-type spines. Our findings are similar to an earlier study wherein E2 was shown to increase mushroom-type spines but not the other spine types in hippocampal neurons (Li et al., 2004), and also the reports showing recruitment of newly synthesized AMPAR to mushroom-type spines where spine size is being determined by the synaptic insertion of GluR1 (Kopec et al., 2007; Matsuo et al., 2008).

Acute application of soluble Aβ oligomers impaired CaMKII, ERK, and Akt/protein kinase B (PKB) activation in mature hippocampal culture (Townsend et al., 2007). As these activated kinases are key players for induction and/or maintenance of LTP as well as spine growth, inhibition of these processes by soluble Aβ oligomers could lead to dysfunction of structural and functional synaptic plasticity. Because soluble Aβ oligomers inhibited CaMKII activation, blocked onset of LTP, and
inhibited phosphorylation of GluR1 at its CaMKII, we tested the possibility that E2 can ameliorate Aβ-induced inhibition of CaMKII-dependent phosphorylation of GluR1. The current data substantiates this possibility, providing direct evidence that E2 has the potential for ameliorating Aβ-induced inhibition of CaMKII mediated phosphorylation and loss of GluR1 surface expression. Electromicroscopic studies show a positive correlation between spine size, synapse number, and synaptic AMPAR number (Takumi et al., 1999). Thus E2-induced increments of surface expression of GluR1 may mechanistically link new mushroom-type spine formation as observed in our experiments.

Decades of research in both humans and animals has provided evidence that E2 is a strong booster for synaptic plasticity and memory. Investigating E2-induced signaling molecules that target synaptic plasticity machinery will not only provide the molecular mechanisms of synaptic plasticity that correlate with memory, but will also provide a therapeutic strategy that may ameliorate age and AD-related dementia.
REFERENCES


ACUTE ESTROGEN TREATMENT AMELIORATES SOLUBLE AMYLOID-BETA PROTEIN OLIGOMER-INDUCED SYNAPTIC DYSFUNCTION

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CHAPTER IV. ACUTE ESTROGEN TREATMENT AMELIORATES
SOLUBLE AMYLOID-BETA PROTEIN OLIGOMER-INDUCED SYNAPTIC
DYSFUNCTION

ABSTRACT

Soluble amyloid beta (Aβ) oligomers cause synaptic dysfunction by inhibiting phosphorylation of CaMKII and AMPAR, important steps involved in structural and functional synaptic plasticity. We found that acute estrogen treatment in vivo and in hippocampal slice cultures ameliorated Aβ oligomer-induced inhibition of CaMKII and AMPAR phosphorylation, reduction of dendritic spine density, and abnormalities in LTP-induced spine growth. These results suggest that acute estrogen treatment has the potential to prevent Aβ oligomer-induced synaptic dysfunction.

Key words: 17β-estradiol, amyloid-beta, CaMKII, AMPAR
INTRODUCTION

The amyloid-beta (Aβ) hypothesis proposes that continuous disruption of normal synaptic function by soluble Aβ oligomers contributes to the development of Alzheimer’s disease (AD). Aβ oligomer-induced alterations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in neural circuits of AD brains (Terry et al., 1991). It has recently been shown that naturally secreted or oligomers isolated directly from the cerebral cortex of subjects with AD, effectively inhibited LTP, enhanced LTD, and reduced dendritic spine density in a normal rodent hippocampus (Shankar et al., 2008). In the same study, the authors also reported that Aβ dimers disrupted the memory of learned behavior in normal rats. In hippocampal slice culture, Aβ oligomer perfusion resulted in activity-dependent inhibition of phosphorylation of CaMKII and AMPAR (GluR1 subunit) [Zhao et al., 2004]. Aβ antibodies and small molecules like 3-amino-1-propane-sulfonic acid (Alzhmed) and scyllo-cyclohexane (hexol AZD-103), prevent Aβ monomers from undergoing oligomerization, improve memory impairment, prevent LTP deficits, and rapidly increase structural plasticity (Walsh and Selko, 2007; Spires-Jones et al., 2009). However, a recent immunotherapy follow-up indicated that treatment failed to improve cognitive deficits in late stage AD patients (Holmes et al., 2008). To date only one small molecule compound, namely Alzhmed, can inhibit plaque formation in amyloid precursor protein (APP) transgenic mice (Gervais et al.,
2006), and is currently in phase III clinical trials (Neurochem. Inc.). A small molecule that has the potential to reverse the AD phenotype in a mouse model is 17β-estradiol (E2). It has been demonstrated that E2 protects against the development of AD-like pathology in transgenic AD mice by preventing Aβ accumulation, tau hyperphosphorylation, and hippocampal-dependent behavioral impairments (Carroll and Pike, 2008). Although other laboratories have reported that LTP and AMPAR function are impaired in transgenic AD mice, no one has directly tested whether E2 reversal of AD phenotype is correlated with the reversal of structural and functional synaptic plasticity dysfunction caused by soluble Aβ oligomers.

**MATERIALS AND METHODS**

**Slice Preparation**

The hippocampus encompassing the CA3-CA1 region was dissected out and 360 µm thick slices were prepared from postnatal day 5 (P5) male C57BL/6 mice in oxygenated artificial cerebral spinal fluid (ACSF) solution using a vibrotone. Immediately after cross-sectioning, organotypic hippocampal slices were maintained as roller-tube cultures as described by Gahwiler, 1981. Cultures were grown in steroid deficient and phenol-red free neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen, Carlsbad, CA). 3-4 days after culturing, these slices were used for the experiments described.

**cLTP Induction**

For cLTP experiments, hippocampal slices were first washed with ACSF [mM: Sucrose 206, KCl 2.8, CaCl 1, NaH₂PO₄ 1.25, NaHCO₃ 26, D-glucose 10, Sodium
Ascorbate 0.4, pH 7.4], then cLTP was induced by adding 10 µM picrotoxin and 200 µM glycine (Lu et al., 2001). It is noted in this publication that the selective activation of synaptic NMDA receptors was achieved by briefly (3 min) elevating the concentration of the coagonist glycine in the perfusion solution to supersaturating levels (100 or 200 µM). The potential activation of glycine receptors was avoided by including strychnine in all of the solutions. Glycine rapidly increased the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs, events that are the consequence of the release of just one or two quanta of transmitter from each terminal), and their NMDA component was dramatically enhanced.

**Soluble Aβ Oligomer Preparation**

Synthetic Aβ₁₋₄₂ (Tocris, Ellisville, Missouri) was prepared without the fibrillar component according to the protocol described by Lambert et al., 2001. In brief, Aβ₁₋₄₂ was dissolved in anhydrous dimethyl sulfoxide (DMSO) to 5 mM, which was then added to ice cold neurobasal medium without phenol-red to 100 µM. This solution was incubated at 4°C for 24 hours, and then centrifuged at 14,000g for 10 minutes. The supernatant comprised of fibrillar-free oligomers, as well as, monomers, was used for the experiments performed in this study. Protein concentration was determined from the supernatant and its molarity was calculated (0.5 µM).

**Western Blot Analysis**

To measure cLTP-induced Aβ-mediated changes in protein phosphorylation, 3 slices for each experimental treatment were used for western blot analysis. After the respective treatments, the individual slices were homogenized in 100 µL of ice cold
buffer containing 50 mM Tris, 10 mM Mg\(^{2+}\), 1 mM EDTA, 1 mM EGTA, 10 mM benzamide, 100 ng/ml leupeptin, 100 ng/ml aprotinin, 0.08 mM sodium molybdate, 0.01% tritonX-100, 10 \(\mu\)M okadaic acid, and 2 mM sodium pyrophosphate, pH 7.4. Aliquots of the lysed and sonicated homogenate were taken to determine protein concentration using protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Samples containing 30 \(\mu\)g protein were electrophoresed on a SDS/PAGE gel. The protein was transferred onto PVDF membrane (Millipore, Billerica, MA), blocked for 1 hour with PBS containing 4% non-fat dried milk, and probed overnight at 4\(^{\circ}\)C with primary antibody. A polyclonal antibody against the alpha subunit of CaMKII phosphorylated at threonine 286 (Cell Signaling Technology, Danvers, MA) was used at a dilution of 1:1000 to detect activation of the kinase (autophosphorylation). A monoclonal antibody against ERK phosphorylated at tyrosine 204 (Santa Cruz, CA) was used at a dilution of 1:1000 to detect activation. A polyclonal antibody was used at a dilution of 1:1000 to recognize GluR1 phosphorylation at the CaMKII site (serine 831, Upstate, Temecula, CA). After washing 3 times with PBS, the membranes were further incubated at room temperature with horseradish peroxidase conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA) at a dilution of 1:1000. The proteins were visualized with supersignal chemiluminesence (Pierce Biotechnology, Rockford, IL) using UVP software (Upland, CA). For the loading control, membranes were stripped and reprobed with total antibody. Antibodies for total CaMKII, total ERK, and total GluR1 were used at a dilution of 1:1000.
**Statistical Analysis**

Densitometric analysis of western blots was conducted using LabWorks Image Acquisition and Analysis software (UVP, Inc., Upland, CA). Densitometric data from at least three independent experiments will be subjected to ANOVA, followed by Newman-Keuls Multiple Comparison Test for the assessment of group differences, and was presented as a bar graph depicting the average ± SD, using GraphPad Prism software (La Jolla, CA). * p<0.05, ** p<0.01, *** p<0.001.

**Icv Injection**

The well established icv injection protocol was used as described by Lu et al., 2002. Briefly the anesthetized ovariectomized female rats were fixed in the stereotaxic frame, and the subcutaneous tissue over the bregma was anesthetized with Xylocaine. The scalp was incised and a bar hole was drilled in the skull near the right coronal suture, 0.9 mm posterior to the bregma and 1.5 mm lateral to the midline. A needle (30-gage) connected to a 10 µl Hamilton syringe, fixed in the stereotaxic frame 4 mm ventral to the skull surface, was then slowly inserted and 3 µl of either vehicle or Aβ<sub>1-42</sub> oligomer solution was injected slowly at a rate of 1 µl per minute. The needle was kept there for 15 minutes to prevent any leakage from the ventricle, before being removed.

**Live Animal Drug Administration and Tissue Procurement**

Female Charles River Sprague-Dawley rats were maintained in laboratory acclimatization for 3 days before ovariectomy. All animal procedures were reviewed
and approved by the University of North Texas Health Science Center Institutional Animal Care and Use Community. Bilateral ovariectomy was performed 3 weeks prior to subcutaneous drug administration. E2 was dissolved in absolute ethanol and then corn oil (Penta Manufacturing, Airfield, NJ) at a concentration of 320 µg/ml. Ethanol was evaporated by incubation at 50°C overnight. A single subcutaneous injection of E2 (320 µg/kg, 3 hrs.) or vehicle was administered, as described by our laboratory (Yang et al., 2003).

**Production of Recombinant Adeno-Associated Virus 2 and Infection of Hippocampal Slice Culture**

The helper plasmid pDG, which contains both the AAV2 genes (rep and cap) and helper genes, (Grimm et al., 1998) were used to generate recombinant AAV2. Recombinant AAV2 vectors were packaged, purified, concentrated, and titered by the following method: Zolotukhin et al., 1999. Recombinant GFP containing AAV2 particles were used at a multiplicity of infection (MOI) of 100. For spine structure analysis the hippocampal slices were incubated with AAV2-GFP containing viruses and grown for 3 days for the expression of GFP in hippocampal neurons.

**Dendritic Spine Density and Spine Structure Analysis**

GFP-expressed hippocampal slices were used for treatment with soluble Aβ oligomers (0.05 µM) for 24 hours prior to stimulation with cLTP. cLTP-induced rapid spine structure alteration was studied in Aβ oligomer treated, untreated, and in the presence or absence of E2 (10 nM) for 1 hour in cLTP induction media as described above. For the spine structure and spine density analysis the hippocampal
slices expressing GFP were used for live cell imaging while submerged in ACSF. Whole pyramidal neurons were imaged by two-photon laser scanning microscopy using Zeiss LSM510 with wide tunable, mode locked, TiSapphire Laser (1.5W Camelon-XR, Coherent, Inc., CA). 910nm excitation was used for imaging GFP. Images of individual CA1 green (GFP) pyramidal neurons were used to make filament objects, spines, and dendrites with Imaris XT software (Bitplane, Saint paul, MN). From the traces, stubby, mushroom and thin type spines were sorted using MatLab XT software (Bitplane, Saint paul, MN). Finally, spines were classified by using “classify spines” program from the MatLab software and this was displayed as color coded spine heads, for example red, blue, and green as thin, mushroom, and stubby respectively.

RESULTS AND DISCUSSION

Therefore, we first asked whether acute E2 treatment has the potential to ameliorate Aβ-induced inactivation of CaMKII and ERK, which are known to be inhibited by soluble Aβ oligomers. To study the activity-dependent effects of E2 and Aβ, we used a protocol intended to induce activation of presynaptic and postsynaptic neurons in organotypic hippocampal slices leading to a long-lasting potentiation of synaptic transmission. This protocol known as chemically-induced long-term potentiation (cLTP) produces activation patterns in all cells of organotypic slice cultures, including both CA1 and CA3 regions. By using this protocol it has been shown that soluble Aβ treatment in hippocampal neurons disrupted activation of CaMKII, ERK, and Akt/PKB (Townsend et al., 2007). Acute hippocampal slices
prepared from normal mice showed Aβ-mediated cLTP-induced inhibition of phosphorylation of CaMKII (cLTP, 223 ± 25%, compared to cLTP and Aβ, 124 ± 14, n=3, p<0.001). E2 treatment increased phosphorylation of CaMKII (cLTP and E2, 373 ± 30%, compared to cLTP, 223 ± 25%, n=3, p<0.001), and E2 treatment ameliorated Aβ mediated cLTP-induced inhibition of phosphorylation of CaMKII (cLTP, E2, and Aβ, 325 ± 50%, compared to cLTP and Aβ, 64 ± 24%, n=3, p<0.001).

Next we studied ERK activity by measuring its phosphorylation status. Similar inactivation patterns were seen with ERK when compared to CaMKII, as shown in Figure 1B. (cLTP, E2, and Aβ, 274 ± 20%, compared to cLTP and Aβ, 68 ± 8%, n=3, p<0.001). AMPAR (GluR1 subunit) at the CaMKII site (serine 831) but not the PKA site (serine 845), is known to be required for LTP induction and memory formation (Lee et al., 2000 and 2003; Whitlock et al., 2006). It has been shown that acute application of Aβ1-42 peptide inhibited phosphorylation by CaMKII (serine 831), but not by PKA (serine 845) [Zhao, 2004]. To see if Aβ-induced inhibition of GluR1 phosphorylation at the CaMKII site can be ameliorated by E2 treatment, we assayed the phosphorylation state of GluR1 in hippocampal slices after cLTP induction. cLTP increased phosphorylation of GluR1 at the CaMKII site (Figure 1C., 231 ± 25%, compared to control, n=3, p<0.001). Aβ inhibited cLTP-induced phosphorylation (cLTP and Aβ, 78 ± 25%, compared to cLTP, 231 ± 25%, n=3, p<0.001), and E2 ameliorated Aβ-mediated cLTP-induced inhibition of GluR1 phosphorylation (Figure 1C., cLTP, E2, and Aβ, 232 ± 34%, compared to cLTP and Aβ, 78 ± 25%, n=3, p<0.001). Activation of CaMKII, as well as phosphorylation of GluR1 at CaMKII
sites, are not only necessary and sufficient for induction of LTP (Lisman et al., 2002), but also for activity-dependent spine structure plasticity (Maletic-Savatick et al., 1999; Jourdain et al., 2003; Matsuzaki et al., 2004; Hayashi et al., 2000). Because CA3-CA1 connections are extensive and presynaptic spontaneous activity is sufficient to fulfill the activity requirement needed for phosphorylation of CaMKII, ERK, and GluR1, we reasoned that in vivo E2 and Aβ treatments would have similar effects with regard to phosphorylation of plasticity specific molecules. To see similar activity dependent Aβ-induced inactivation of CaMKII and ERK in vivo, we injected synthetic Aβ1-42 oligomers (0.5 µM) into the intracerebral ventricle (icv) of ovariectomized rats, and 24 hrs. after injection, the rats were dosed with E2 (320 µg/kg, 3 hrs., subcutaneous injection). We subsequently assessed the phosphorylation state of CaMKII, ERK, and GluR1. In vivo studies revealed that like in organic hippocampal slices, E2 increased the phosphorylation of CaMKII (366 ± 60%, compared to control, n=3, p<0.01), ERK (256 ± 62%, compared to control, n=3, p<0.01), and GluR1 (211 ± 49%, compared to control, n=3, p<0.01), while Aβ treatment inhibited their phosphorylation (46 ± 13%, 50 ± 20%, 30 ± 3%, compared to control, respectively) [Figure 1D., 1E., and 1F.]. E2 was also able to ameliorate the inhibitory effects of Aβ1-42 oligomers (CaMKII, 244 ± 69%, ERK, 207 ± 45%, GluR1, 200 ± 37%, compared to Aβ) [Figure 1D., 1E., and 1F.]. E2 has been shown to modulate hippocampal spine structure plasticity in vivo in association with an enhanced performance in hippocampal-dependent cognitive tasks (Li et al., 2004). To examine whether E2-mediated activation of CaMKII and/or increased
phosphorylation of GluR1 correlates with the changes in Aβ-induced synaptic architecture and/or synaptic loss, we analyzed spine morphology and spine density in adeno-associated virus containing green florescent protein (AAV2-GFP)-infected pyramidal neurons from organotypic mouse hippocampal slices. Pyramidal neurons in slices cultured for 4 days with AAV2-GFP virus, were then subsequently treated with Aβ_{1-42} for 24 hrs., and finally were activated with the cLTP induction protocol for 1 hr. Whole pyramidal neurons were imaged by two-photon laser-scanning microscopy. 910 nM excitation was used for imaging GFP (Supplemental Figure 1, top panel). These cLTP activated and Aβ_{1-42} exposed pyramidal neurons showed a reduced amount of mushroom type spines without affecting total spine density (Figure S1A. compared to Figure S1C.-bottom panel, 280 vs. 190 mushroom spines [representation of one neuron for each treatment, Figure S1A. and S1C.]). E2 treatment increased mushroom spines compared to cLTP (cLTP mushroom spines, 280, compared to cLTP and E2, 460 mushroom spines). Aβ-mediated cLTP-induced inhibition of mushroom-type spine number was ameliorated by E2 treatment (Figure S1D. and S1C.-bottom panel, cLTP, E2, and Aβ, 350 mushroom spines, compared to cLTP and Aβ, 190 mushroom spines). Detailed analysis as shown in figure 2, revealed that synthetic Aβ caused a 50% decrease in mushroom-type spine density and E2 ameliorated this decrease. Taken together, our results show that activity-dependent Aβ-induced inactivation of CaMKII and ERK, Aβ-induced decreased phosphorylation of GluR1, as well as, decreased number of excitatory synapse specific spine number (mushroom type) were ameliorated by E2. The molecular
mechanism by which E2 ameliorated Aβ-induced dysfunction of structural and functional plasticity, is not completely understood. Previously we have shown that E2 directly potentiates L-type VGCC in hippocampal neurons (Sarkar et al., 2008). L-type VGCC-mediated activation of CaMKII (Lee et al., 2009; Matsuzaki et al., 2004) and NMDAR activation of Ras-ERK (Harvey et al., 2008) at the synapse, rapidly modulate structural and functional synaptic plasticity. Thus, our results raise the possibility that E2 by potentiating L-type VGCC activates CaMKII and ERK at the synapse, which has the potential to ameliorate Aβ-induced synaptic dysfunction.
Aβ\textsubscript{1-42} oligomers inhibit and acute E2 rapidly ameliorates neuronal activity-dependent phosphorylation of CaMKII, ERK, and GluR1 in hippocampal neurons. cLTP was used to activate organotypic slice cultures for 1 hr. in presence or absence of soluble Aβ\textsubscript{1-42} oligomers (0.5 µM) and E2 (10 nM), and analyzed using western blot. cLTP-induced phosphospecific signal of CaMKII (serine 286) normalized to total CaMKII (Figure 1A.), pERK (tyrosine 42 and 44) normalized to total ERK (Figure 1B.), and pGluR1 (serine 831) normalized to total GluR1 (Figure 1C.), showed a significant increase in phosphorylation, but Aβ inhibited the induction of their phosphorylation (Figure 1A.-1C.). E2 ameliorated Aβ-mediated cLTP-induced inhibition of phosphorylation of the above mentioned proteins (325 ± 50%, 274 ± 20%, 232 ± 34%, respectively). Subcutaneous in vivo E2 (320 µg/kg, 3 hrs.) treatment in ovariectomized rats induced phosphospecific signal of CaMKII, ERK, and GluR1 (Figure 1D., 1E., and 1F.). In vivo Aβ oligomer (0.5 µM) exposure to the hippocampus by icv injection to ovariectomized rat brains inhibited the phosphorylation of CaMKII (Figure 1D.), ERK (Figure 1E.), and GluR1 (Figure 1F.). E2 treatment ameliorated Aβ-mediated inhibition of phosphorylation of the above mentioned proteins (CaMKII, 244 ± 69%, ERK, 207 ± 45%, GluR1, 200 ± 37%, compared to Aβ) [Figure 1D., 1E., and 1F.]. Data are mean ± SD, one-way ANOVA, * p<0.05, ** p<0.01, *** p<0.001, n=3.
C. pGluR1
GluR1

D. pCaMKII
CaMKII
E. pERK/ERK

F. pGluR1/GluR1
FIGURE 2

Analysis of Spine Density and Spine Type in Pyramidal Neurons from Hippocampal Slices. Bar graph showing quantitative analysis of spine counts/µm dendritic length. E2 (10 nM) increased mushroom- and long-thin-type spine density (green and blue) [0.35 ± 0.03 spines/µm and 0.77 ± 0.035 spines/µm, respectively]. Soluble Aβ1-42 oligomer (0.5 µM) treatment inhibited mushroom-type spine density (0.07 ± 0.005 spines/µm) without affecting other spine densities (stubby and long-thin, red and blue). E2 (10 nM) treatment ameliorates Aβ-induced mushroom-type spine density loss (0.15 ± 0.01 spines/µm). Data are mean ± SD, **, p<0.01, ***, p<0.001, two-way ANOVA test, n=6 (3 independent experiments).
SUPPLEMENTAL FIGURE 1

$A\beta_{1-42}$ oligomers rapidly decrease and E2 ameliorates cLTP-induced mushroom-type spine density. Hippocampal slices from mice (P5) were infected with AAV2-GFP viruses. 4 days after post-infection, slices were cLTP-induced in the presence or absence of soluble $A\beta_{1-42}$ (0.5 µM) and E2 (10 nM). Immediately after treatment, two-photon confocal images of live slices were acquired. Whole pyramidal neurons were imaged by two-photon laser-scanning microscopy. 910 nM excitation was used for imaging GFP (A. cLTP, B. cLTP and E2, C. cLTP and $A\beta$, and D. cLTP, E2, and $A\beta$). Respective images were also used for spine density analysis. Using Imaris XT software, neurons were filament traced, spines were assigned, and then spines were classified as stubby (red), mushroom (green), and long-thin (blue), using Imaris XT/MatLab spine classifier. Respective two-photon micrograph showing assigned spines in the whole neuron (A., B., C., and D.), cropped distal dendrites showing assigned spines, and their respective spine counts shown in bar graphs.
B. cLTP + E2

[Image: Diagram showing spine counts for different categories: Stubby, Mushroom, Long Thin]

Spine Counts

[Bar chart showing comparative spine counts for Stubby, Mushroom, and Long Thin categories]
C. cLTP + Aβ

[Image of neuronal structures and a bar graph showing spine counts for different types of spines.]
D. cLTP + E2 + Aβ
REFERENCES


CHAPTER V. SUMMARY AND FUTURE DIRECTION

SUMMARY

The main goal of this project was to determine 1) the mechanism of action by which E2 potentiates the L-type VGCC and 2) estrogen-induced signaling which links structural and functional synaptic plasticity.

It is well documented that estrogen acutely alters the intrinsic and synaptic physiology of neuronal circuits (within minutes) in various brain regions. For example, the electrophysiological effects of estrogen in the hippocampus serve to facilitate neuronal firing, increase excitatory synaptic transmission, and increase the capacity for synaptic plasticity, which includes both LTP and LTD. Activity-dependent influx of calcium in neurons (via NMDARs and/or L-type VGCC) leads to persistent neuronal activity, induction of synaptic plasticity (LTP and LTD), and activation of kinases and transcription factors important for synaptic plasticity. However, the molecular and cellular mechanisms by which the acute effects of estrogen on electrophysiology are coupled to plasticity and memory are still not fully understood. Our data suggests a new possible mechanism by which estrogen, via L-type VGCC potentiation, modulates memory related synaptic plasticity. The very rapid onset of E2 action, which is less than a second, supports a direct interaction of E2 with the channel protein. This idea was confirmed by experiments such as: whole
cell binding assay in HT-22 cells, whole cell binding in HEK 293 cells transiently expressed with wild-type and mutant $\alpha_{1c}$ channels, competitive binding assay using radioligand, and electrophysiological studies using wild-type and DHP insensitive channels. E2’s ability to displace Bay K 8644 (an L-type VGCC agonist) also provides evidence for its direct mechanism of action. Our studies confirm that estrogen itself binds with high affinity to the L-type VGCC, at a domain that overlaps with the DHP site.

To understand whether E2-induced biochemical signaling mechanistically links synaptic plasticity, we determined the phosphorylation patterns of structural and functional plasticity related proteins (AMPAR [GluR1 subunit], CaMKII, and ERK). We found that estrogen rapidly increased phosphorylation of CaMKII, ERK, and AMPAR (GluR1 subunit) in primary cortical neurons and in vivo (cortex). CaMKII inhibitor, KN-93, inhibited phosphorylation levels of GluR1 in primary cortical neurons. We also determined that soluble Aβ$_{1-42}$ oligomers abrogated, while E2 ameliorated phosphorylation of GluR1 at its CaMKII site. Aβ treatment also inhibited GluR1 trafficking, but E2 prevented this inhibition in cortical neurons. Our data determined that estrogen-induced signaling does in fact mechanistically link structural and functional plasticity, because we observed that E2 treatment rapidly increased spine number and ameliorated Aβ-induced spine loss.

In comparison with the primary cortical neuronal and in vivo cortical data, we found that acute estrogen treatment in hippocampal slice culture and in vivo in the hippocampus ameliorated Aβ oligomer-induced inhibition of CaMKII and AMPAR
phosphorylation, reduction of dendritic spine density, and abnormalities in LTP-induced spine growth. Taken together, these results suggest that acute estrogen treatment has the potential to prevent Aβ oligomer-induced synaptic dysfunction.

**FUTURE DIRECTION**

Molecular mechanisms by which estrogen activates CaMKII and ERK, has yet to be elucidated. Moreover, the experimental results which were shown in this project did not address the question of whether estrogen receptors (ERs) are involved in the activation of these kinases. Very recently it has been shown that CaMKII in spines was specifically activated by NMDARs and L-type VGCC, presumably by a nanodomain of Ca\(^{2+}\) near the channels, in response to glutamate uncaging (Matsuzaki et al., 2004) and depolarization (Lee et al., 2009), respectively. Based on this data we reasoned that one potential mechanism by which estrogen may activate the above mentioned kinases is by potentiating the L-type VGCC. Also, it has been reported that estrogen-induced potentiation of L-type VGCC does not require estrogen receptor activation (Sarkar et al., 2008). Our data supports this possibility and the proposed future studies will test this hypothesis.

To study the role of ERα and ERβ in activating CaMKII and ERK, we will knock-down ERα and ERβ in primary neuronal culture using small interfering ribonucleic acid (siRNA) technology. Specifically we will first design the oligonucleotides encoding the coding regions of ERα and ERβ, using siDirect software program (online design sight). These oligos will then be inserted in front of the RNA polymerase III promoter, resulting in ER siRNA expression cassette. This
cassette will be cloned into a site flanked by the AAV2 terminal repeats. Downstream of the siRNA expression cassette a GFP expression cassette under the polymerase II promoter will be inserted into that construct, resulting in a plasmid DNA that can be packaged into AAV2 viruses. This virus will be used for infecting primary cortical neurons, and effects of knocking down estrogen receptors will be assessed by measuring the estrogen-induced activation of CaMKII and ERK. Alternatively, the role of ERα and ERβ in estrogen-induced activation of CaMKII and ERK can be studied in ERα and ERβ knock-out mice. These mice are commercially available or can be obtained upon request for the above mentioned studies.

Our hypothesis is that estrogen by potentiating L-type VGCC can increase Ca\(^{2+}\) influx at postsynaptic sites which subsequently leads to the activation of CaMKII and ERK. This will be tested by the following experiments: 1) Primary neuronal culture will be treated with various concentrations of L-type VGCC inhibitor (nifedipine), and the phosphorylation state of these kinases will be assessed, 2) siRNA mediated knocking-down of L-type VGCC in primary neuronal culture will be used to determine whether estrogen can affect activation of kinases in these cells, and 3) In vivo role of L-type VGCC in estrogen-induced activation of CaMKII and ERK will be studied by subcutaneous injection of E2 in brain specific L-type VGCC knock-out mice. The proposed studies will provide information on the molecular mechanism by which estrogen activates plasticity related kinases. Moreover, if these studies confirm that estrogen activation of plasticity related kinases is in fact nongenomic, the use of nonfeminizing E2 derivatives (ZYC compounds) looks more
promising as a potential therapeutic for the treatment of AD. Because ZYC compounds do not bind to either ERα or ERβ (pM to µM range) [Perez et al., 2006] nor do they stimulate uterine growth in ovariectomized rats (Perez et al., 2005), adverse side effects once known to accompany hormone therapy are eliminated.
REFERENCES


CHAPTER VI. APPENDIX

FIGURE 1

Cell viability of neuronal culture. Hippocampal neurons were grown in neurobasal medium (minus phenol red) supplemented with B27 and pen-strep for 7 days. A) Phase contrast image of hippocampal neurons. B) Cell viability was determined by calcein acetoxyethyl (AM) assay and visualized with ultraviolet microscopy.
FIGURE 2

Purity of neuronal culture determined by staining with microtubule-associated protein 2 (MAP2) [red] and glial fibrillary acidic protein (GFAP) [green]. Hippocampal neurons were maintained for 7 days in conditions similar to those described under Figure 1, prior to dual staining and visualization with laser scanning confocal microscopy. All images are taken from the same field of view. A) Fluorescence image of hippocampal neurons stained for MAP2. B) Fluorescence image of hippocampal neurons stained for GFAP. C) Merge of (A) and (B). Note all cells stain positive for MAP2 (A) in comparison to cells staining positive for GFAP (B), indicating a 99% pure neuronal culture.
FIGURE 3

E2 induces phosphorylation of CaMKII and ERK in the cell body and dendritic extensions of primary cortical neurons. Cortical neurons untreated or treated with E2 (10 nM) for either 20 min. or 30 min. were labeled with A.) phospho-CaMKII and 4',6-diamidino-2-phenylindole (DAPI) or B.) phospho-ERK and DAPI respectively and visualized by fluorescence microscopy.

A) pCaMKII

Control

E2

B) pERK

Control

E2
FIGURE 4

Acute E2 treatment increases phosphorylation of CaMKII, ERK, and GluR1 in hippocampal neurons in vivo. Subcutaneous injection (320 µg/kg) of E2 in ovariectomized rats significantly increased phosphorylation of A) CaMKII, B) ERK, and C) GluR1 6 hrs. after injection, and all rapidly returned to their respective basal levels at 24 hrs. Bar graphs are relative density of A) pCaMKII, B) pERK, and C) pGluR1 bands compared to control (assigned as 100%). Data are ± SD. **, p<0.01, ***, p<0.001, one-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=3 (3 independent experiments).
FIGURE 5

Estrogen-induced phosphorylation of GluR1 (serine 845) was not inhibited by CaMKII inhibitor, KN-93, in primary cortical neurons. Primary cortical neurons (E18) were grown 15 days in vitro (DIV) and treated with E2 (10 nM) in the presence or absence of CaMKII inhibitor (KN-93, 5 µM), MEK inhibitor (UO126, 10 µM), and PI3K inhibitor (LY29402, 10 µM) for 30 and 60 min. Induction of phosphorylation of GluR1 at serine 845 was not inhibited by KN-93, U0126, or LY29402 compared to 60 min. vehicle-treated (DMSO) control. Data are mean ± SD. *, p<0.5, **, p<0.01, two-way ANOVA test, Newman-Keuls Multiple Comparison Test, n=3 (3 independent experiments).