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Modulation of α CaMKII signaling by rapid ER α action

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Abstract

The estrogen receptor (ER) subtypes, ER α and ER β , modulate numerous signaling cascades in the brain to result in a variety of cell fates including neuronal differentiation. We report here that 17 β -estradiol (E2) rapidly stimulates the autophosphorylation of α -Ca²⁺/calmodulin-dependent kinase II (α CaMKII) in immortalized NLT GnRH neurons, primary hippocampal neurons, and Cos7 cells co-transfected with ER α and α CaMKII. The E2-induced α CaMKII autophosphorylation is ER α - and Ca²⁺/calmodulin (CaM)-dependent. Interestingly, the hormone-dependent association of ER α with α CaMKII attenuates the positive effect of E2 on α CaMKII autophosphorylation, suggesting that ER α plays a complex role in modulating α CaMKII activity and may function to fine-tune α CaMKII-triggered signaling events. However, it appears as though the activating signal of E2 dominates the negative effect of ER since there is a clear, positive downstream response to E2-activated α CaMKII; pharmacological inhibitors and RNAi technology show that targets of ER α -mediated α CaMKII signaling include extracellular signal-regulated kinase 1/2 (ERK1/2), cAMP response element-binding protein (CREB), and microtubule associated protein 2 (MAP2). These findings suggest a novel model for the modulation of α CaMKII signaling by ER α , which provides a molecular link as to how E2 might influence brain function.

Classification term

Intracellular signal transduction

Keywords

rapid estrogen action; estrogen receptor; Ca²⁺/calmodulin-dependent protein kinase II; phosphorylation; signal transduction

Introduction

Estrogen is intimately involved in the development and regulation of a variety of tissues, including the brain where it functions as a potent neuroprotective agent (Tang, 1996; Sooter, 1999) and a positive influencer of mood and cognition in both humans and rodents (Duff, 2000; Ahokas, 2001; Estrada-Camarena, 2003; Sandstrom, 2004; Almeida, 2005). Specifically, estrogen replacement in ovariectomized female rats significantly improves spatial

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memory retention as tested by a delayed matching-to-place water maze task (Sandstrom, 2004), and examination of the ER α knock-out mouse model (ER α KO) demonstrated that ER α is required for learning hippocampal-dependent inhibitory avoidance tasks (Fugger, 2000). Clearly, a connection between estrogen and brain function exists, however, the underlying mechanisms are still an area of fierce investigation.

Estrogen influences cognition primarily by its rapid modulatory effects on multiple signaling pathways. The rapid effects of estrogen refer to those events that occur seconds to minutes after estrogen administration and are not sensitive to inhibitors of transcription or translation (Heldring, 2007). Wu (2005) as well as Zhao (2005) have shown that E2 induces Ca²⁺ influx in hippocampal neurons within seconds, initiating signaling cascades that result in varied neurotrophic responses including neurite outgrowth and neuroprotection. E2 also rapidly induces c-Src activation in explanted mouse cortical neurons to influence neuronal differentiation (Nethrapalli, 2001). CREB phosphorylation is stimulated by E2 through the activation of ERK1/2 in forebrain cholinergic neurons, which requires ER α but not ER β (Szego, 2006). Additionally, Sawai (2002) reported that E2 induces α CaMKII activity in hippocampal neurons in an ICI-sensitive manner, implicating the involvement of ER; however, the mechanism was not addressed.

α CaMKII plays an essential role in neuronal differentiation and cognitive processes (Silva, 1992a; Blanquet, 1999; Gaudilliere, 2004), thus defining the relationship between E2 action and α CaMKII signaling is imperative for a better understanding of the mechanism by which E2 exerts its effects on the brain. α CaMKII is a multi-subunit serine/threonine kinase that is exquisitely responsive to Ca²⁺ levels (Hanson, 1992). Under basal conditions, α CaMKII is kept inactive by its autoinhibitory domain and only the binding of Ca²⁺/CaM to this domain disrupts autoinhibition, allowing for kinase activity. Autophosphorylation of α CaMKII at residue Thr286 occurs when the duration or magnitude of the Ca²⁺ signal increases and two neighboring kinase subunits bind Ca²⁺/CaM. This permits the activation of one subunit while the second subunit undergoes a conformational change, revealing Thr286 to the active subunit for phosphorylation. CaMKII remains active until it is dephosphorylated, even when Ca²⁺ levels return to normal (Hanson, 1992; Hudmon, 2002).

In the present study, the hypothesis that CaMKII signaling is modulated by ER action is investigated. We show that E2 rapidly induces the autophosphorylation of α CaMKII in an ER α - and Ca²⁺ influx-dependent manner in transfected Cos7 cells, immortalized GnRH neurons, and primary hippocampal neurons. Interestingly, the physical interaction of ER α with α CaMKII counteracts the positive effect of E2 on α CaMKII activity suggesting a dual role for ER α in modulating α CaMKII function. Ultimately, the E2-evoked α CaMKII activity results in ERK1/2, CREB, and MAP2 phosphorylation in primary hippocampal neurons.

Results

E2 rapidly induces α CaMKII autophosphorylation in the cytoplasm of NLT cells via a Ca²⁺-dependent mechanism

To examine the effect of E2 on α CaMKII activation it was first established that ER α is expressed in NLT immortalized GnRH neurons via immunofluorescence (Fig. 1A) and western blot analysis (Fig. 1B). Additionally, a luciferase reporter assay in which an estrogen response element (ERE) is linked to luciferase (3ERE-Luc) shows that NLT cells transfected with the 3ERE-Luc reporter plasmid have significantly increased luciferase activity when treated with either E2, propyl pyrazole triol (PPT: ER α -selective agonist), or diarylpropionitrile (DPN: ER β -selective agonist) but not with vehicle or in the presence of ICI 182, 780 (ICI: potent ER α / β antagonist), confirming that NLT cells express functional ER α and ER β (Fig. 1C). Treatment of NLT cells with increasing concentrations of E2 results in a dose-dependent

increase of α CaMKII autophosphorylation (Fig. 1D), and subsequent experiments were performed using a 10nM concentration of E2 unless otherwise stated. The autophosphorylation of α CaMKII was also examined over time, and the peak of E2-induced kinase autophosphorylation occurs at 10 min of treatment. As a positive control a combination of forskolin and A23187 (F/A) was used to ultimately increase intracellular Ca^{2+} levels (Fig. 1E). This effect is blocked by pre-treatment with KN-62, a CaMKII-specific inhibitor, or ICI as shown by Western blot analysis (Fig. 1F).

Immunofluorescence studies demonstrates that in NLT cells, E2 stimulates α CaMKII activation in the cytoplasm and cell outgrowths within 10 min compared to unstimulated cells, and KN-62 pre-treatment counteracts the E2 effect (Fig. 2A). Specifically, E2 induces kinase autophosphorylation in $43.10 \pm 8.49\%$ of cells, whereas only $8.31 \pm 2.88\%$ of unstimulated cells and $20.54 \pm 12.81\%$ of E2+KN-62-treated cells have autophosphorylated α CaMKII in the cytoplasm. In contrast, the nuclear pool of α CaMKII in NLT cells appears to be activated regardless of the treatment. Fractionation of NLT cells after 10 min of ligand treatment confirmed that E2-induced α CaMKII autophosphorylation occurs primarily in the cytosolic fraction, which is considerably decreased by KN-62 (Fig. 2B).

To begin to understand the mechanism by which E2 influences α CaMKII autophosphorylation we examined the hypothesis that Ca^{2+} signaling is involved since E2 rapidly triggers Ca^{2+} influx and mobilization in neuronal cells (Wu, 2005; Zhao, 2005). NLT cells were either unstimulated or treated for 10 min with vehicle, E2 alone, or E2 with 30 min pre-treatment of BAPTA-AM (intracellular Ca^{2+} chelator), W7 (CaM inhibitor), or Nifedipine (L-type voltage-gated Ca^{2+} channel antagonist) (Fig. 2C/D). Western blot analysis demonstrates that E2-induced α CaMKII autophosphorylation is dependent on both intracellular Ca^{2+} and CaM action as it is completely blocked by BAPTA-AM and W7 pre-treatment. Ca^{2+} influx through L-type Ca^{2+} channels is also clearly involved as Nifedipine significantly decreases α CaMKII autophosphorylation induced by E2. Treatment with BAPTA-AM, W7, and Nifedipine alone had no effect on α CaMKII autophosphorylation (data not shown). These data suggest that E2-induced α CaMKII autophosphorylation is the result of Ca^{2+} influx via L-type Ca^{2+} channels, which can then complex with CaM to stimulate α CaMKII activity.

ER α and not ER β is responsible for E2-induced α CaMKII activity

As both ER α and ER β are expressed in a variety of brain regions (Shughrue, 1997) and in NLT cells (Fig. 1C), it is important to understand if one or both of them could be involved in stimulating α CaMKII autophosphorylation. To address this issue, the effect of two ER-selective ligands, PPT (ER α -selective agonist) and DPN (ER β -selective agonist), was examined.

Increasing concentrations of the receptor-selective ligands were applied to NLT cells for 10 min, and while PPT is able to induce α CaMKII autophosphorylation in a dose-dependent manner, DPN is unable to elicit the same response even though the levels of ER α and ER β are relatively similar (Fig. 3A/B). These results suggest that ER β , while transcriptionally active, does not appear to mediate α CaMKII autophosphorylation in these cells. Additionally, E2 treatment is unable to induce α CaMKII autophosphorylation at any of the time points examined in a neuroblastoma cell line (SK-N-SH) (Fig. 3C) shown to be devoid of ER α but express functional ER β as evidenced by luciferase assay (Fig. 3D), which is in agreement with previous reports (Ba, 2004; Wen, 2004). Additionally, Cos7 cells transfected with α CaMKII alone or in the presence of either ER α or ER β , and then treated with E2 confirmed the involvement of ER α . The co-expression of ER α but not ER β with α CaMKII results in increased kinase autophosphorylation within just 2 min of E2 treatment. This effect is blocked by pre-treatment with KN-62 and is non-existent in the presence of vehicle. In the absence of ER α , E2 is unable

to stimulate α CaMKII activity (Fig. 2E/F). These results indicate that ER α mediates the activation of α CaMKII by E2, and ER β is not involved in the examined cellular contexts.

ER α interacts with α CaMKII in a hormone-dependent manner to attenuate E2-induced α CaMKII autophosphorylation

Examination of the α CaMKII amino acid sequence reveals that it contains a nuclear receptor interaction motif, or NR box, within the CaM-binding region of the autoinhibitory domain. If α CaMKII can interact with ER α via this NR box, an LTTML sequence, mutation of the consensus sequence should impair the association. GST pulldown studies show that when the NR box of α CaMKII is disrupted by the mutation L304A, the E2-dependent interaction observed between wild type α CaMKII and GST-ER α -LBD (ligand binding domain) is completely abolished (Fig. 4A). To understand if the kinase binds to ER in a manner similar to that of a typical nuclear receptor coactivator, the ability of α CaMKII to bind various GST-ER α -LBD mutants was examined. It was previously reported that an array of point mutations in the ER α -LBD (ER α -I358R, V376R, and E542K) disrupt the binding of the glucocorticoid receptor interacting protein (GRIP) and steroid receptor coactivator 1 (SRC-1) to the hydrophobic binding pocket of ER α (Feng, 1998). Interestingly, α CaMKII is unable to bind any of the GST-ER α -LBD mutants even in the presence of E2, suggesting that α CaMKII binds to the same hydrophobic pocket as other typical coactivator proteins (Fig. 4B). Additionally, no association is observed in the presence of tamoxifen (T) in Figure 4A because this ligand induces the antagonist conformation of ER α -LBD, thus inhibiting α CaMKII binding.

To test if the two proteins interact in cells, Cos7 cells were co-transfected with FLAG-ER α and α CaMKII, and the proteins were then immunoprecipitated with either anti-FLAG or anti- α CaMKII, respectively. Western blot analysis for ER α and α CaMKII shows that the proteins associate only in cells treated with E2 but not vehicle for 10 min (Fig. 4C). To determine if the interaction with α CaMKII was selective for ER α over ER β , Cos7 cells were co-transfected with FLAG-ER β and α CaMKII, and the proteins were immunoprecipitated with either anti-FLAG or anti- α CaMKII, respectively. Interestingly, in this context, ER β associates with α CaMKII in a hormone-independent fashion as the interaction occurs in the presence of either vehicle or E2 (Fig. 4D). To confirm that the ER α -LBD mutant, V376R, is incapable of interacting with α CaMKII as demonstrated by GST pulldown experiments, Cos7 cells were co-transfected with α CaMKII and either wild-type ER α or ER α -V376R, and ER α was then immunoprecipitated with the ER-specific antibody, H222.2. Expectedly, the mutation of V376R effectively disrupted the E2-dependent interaction between α CaMKII and ER α in cells (Fig. 4E).

Although data in Figure 2C provides evidence that E2-induced α CaMKII autophosphorylation is dependent on Ca²⁺ signaling, it is fair to hypothesize that α CaMKII could also be directly activated by ER α through its interaction with the autoinhibitory region of the kinase, which could potentially disrupt its autoinhibition much in the same manner as calmodulin. To test this hypothesis, Cos7 cells were co-transfected with α CaMKII and either wild type ER α or the interaction mutant, ER α -V376R. The cells were unstimulated or treated with E2, or E2 +BAPTA-AM pre-treatment, and α CaMKII autophosphorylation was detected by western blot analysis (Fig. 4F/G). Surprisingly, disrupting the interaction of ER α with α CaMKII significantly enhances the ability of E2 to stimulate kinase autophosphorylation (V376R expression versus wild-type expression: ~4-fold increase versus ~2-fold increase in autophosphorylation). Additionally, pre-treatment with BAPTA-AM abolishes E2-induced α CaMKII autophosphorylation when the ER α - α CaMKII interaction is disrupted, implying that E2 can still influence Ca²⁺ signaling to activate α CaMKII even when its interaction with ER α is impaired. Since the ER α binding site overlaps with the CaM-binding site, these data suggest the possibility that ER α may compete with Ca²⁺/CaM binding on α CaMKII and

decrease its kinase activity, perhaps by locking the kinase subunits in an autoinhibited state. Taken together, these data show that while E2 can stimulate α CaMKII autophosphorylation via Ca^{2+} influx, the association of ER α with α CaMKII negatively regulates this event, perhaps to keep the amount of active kinase in check.

E2 stimulates ERK1/2 and CREB phosphorylation via a CaMKII-and L-type Ca^{2+} channel-dependent mechanism in NLT neurons

ERK1/2 and CREB have both been shown to play a role in neuronal survival, differentiation, and LTP depending on the stimuli (Winder, 1999; Piiper, 2002; Song, 2005). Furthermore, E2 has been reported to stimulate their activation in various populations of neurons (Lee, 2004; Szego, 2006), although the role of α CaMKII in this activation has not been thoroughly investigated. To examine the effect of E2-induced α CaMKII activity on ERK1/2 and CREB phosphorylation, NLT cells were either unstimulated or treated for 10 min with vehicle, E2, or E2 plus 30 min pretreatment with KN-62, ICI, Nifedipine, or U0126, a commonly used inhibitor of ERK1/2 activity. Western blot analysis shows that E2 treatment rapidly and significantly stimulates ERK1/2 and CREB phosphorylation over control and vehicle-treated cells. This effect is blocked by the inhibition of CaMKII and L-type voltage-gated Ca^{2+} channels, confirming the involvement of both proteins (Fig. 5A/B). E2-stimulated CREB activity is also decreased by blocking ERK1/2 signaling with U0126, indicating that ERK1/2 is upstream of CREB. The involvement of CaM was also examined and 30 min pre-treatment of NLT cells with calmidazolium (Calm), a potent CaM inhibitor, prior to 10 min of E2 completely blocks the effect of E2 on ERK1/2 and CREB phosphorylation (Fig. 5C/D), revealing that CaM action is, in fact, important for E2-induced ERK1/2 and CREB activation.

E2-induced α CaMKII signaling in primary hippocampal neurons

The ability of E2 to signal to α CaMKII as well as downstream target proteins was examined in cultured embryonic primary hippocampal neurons, which were chosen as a model due to the prevalence and functional significance of α CaMKII in these cells, and because we wanted to test our hypothesis in a non-immortalized cell line. Primary hippocampal neurons were either unstimulated or treated for 10 min with vehicle, E2, or E2 plus 30min pre-treatment with KN-62, ICI, or U0126, and western blot analysis was used to detect the phosphorylation status of a variety of proteins (Fig. 6A/B). E2 stimulates the autophosphorylation of α CaMKII as well as the phosphorylation of ERK1/2, CREB, and MAP2. Interestingly, E2 does not induce the phosphorylation of another MAPK family member, ERK5, or the transcription factor, ELK1. Inhibition of either CaMKII or ER activity with KN-62 or ICI, respectively, significantly decreases E2-induced ERK1/2, CREB, and MAP2 phosphorylation, and E2-stimulated CREB and MAP2 phosphorylation is reduced by blocking ERK1/2 action with U0126. Surprisingly, U0126 also inhibits α CaMKII autophosphorylation by E2, implicating MEK1/2 as a positive regulator of this event. Additionally, the effect of Ca^{2+} /CaM signaling was investigated, and figure 7 shows that E2-induced ERK1/2, CREB, and MAP2 phosphorylation is severely compromised when Ca^{2+} signaling and CaM action were disrupted with BAPTA-AM or calmidazolium pre-treatment, respectively.

To directly demonstrate the requirement of α CaMKII for the E2-induced phosphorylation of downstream signaling proteins, its protein expression in primary hippocampal neurons was knocked down using specific siRNA oligonucleotides (Fig. 8A). Targeted inhibition of α CaMKII expression was achieved in si α CaMKII-transfected hippocampal neurons as demonstrated by western blot analysis; the total α CaMKII level is knocked down to ~23% of the level in neurons transfected with non-targeting (N.T.) siRNA (Fig. 8B). The siRNA-mediated inhibition of α CaMKII prevents E2-induced α CaMKII autophosphorylation (Fig. 8C) as well as ERK1/2 (Fig. 8D), CREB (Fig. 8E), and MAP2 (Fig. 8F) phosphorylation to the same extent as KN-62 pre-treatment. Together, these results indicate that α CaMKII action

mediates the E2-induced phosphorylation of ERK1/2, CREB, and MAP2 in cultured embryonic primary hippocampal neurons.

Discussion

The ability of E2 to influence signaling pathways in the brain has been examined with increasing interest however its effect on α CaMKII activity has not been described in detail. α CaMKII is a particularly appealing signaling partner for ER since linking the two proteins would provide valuable insight into the mechanism by which E2 influences cognitive function. We report here that E2 rapidly induces α CaMKII autophosphorylation in an ER α - and Ca^{2+} influx-dependent manner. This signaling is extranuclear and ultimately results in the phosphorylation of a variety of downstream proteins in immortalized GnRH neurons and cultured embryonic primary hippocampal neurons. Interestingly, the association of ER α with α CaMKII negatively impacts the ability of E2 to induce α CaMKII autophosphorylation, suggesting a novel model for the modulation of α CaMKII activity by ER α .

An early indication that ER signaling intersects with that of α CaMKII was first demonstrated in hippocampal neurons where Sawai and colleagues (2002) showed that E2 stimulates the activity of α CaMKII in an ICI-sensitive manner, implicating the involvement of ER. This previous study raises several interesting questions: which ER subtype mediates E2-induced α CaMKII activity and in what cellular compartment does it occur? What is the mechanism by which ER modulates α CaMKII activity? What are the downstream signaling events? We wanted to address these questions as well as others with this work.

We initially studied ER-modulated α CaMKII activity in NLT cells, the immortalized GnRH cell line, and found that the effect of E2 on α CaMKII autophosphorylation was rapid and transient. While E2 allowed for the autophosphorylation of neighboring kinase subunits, it was short-lived, presumably due to the dephosphorylation of the subunits by nearby phosphatases. As such, the stage of long-term/persistent α CaMKII activation by E2 was never reached in NLT cells since the rate of autophosphorylation never exceeded the rate of dephosphorylation. We also used cultured embryonic primary hippocampal neurons as a cellular model because 1) ER α has been found in the hippocampus of rodents and humans (Solum, 2001; Adams, 2002; Hu, 2003), and there are numerous reports detailing the effects of E2 on the rodent hippocampus including its positive role in dendritic spine density and synapse density (Woolley, 1998), and 2) α CaMKII is abundant in the hippocampus and its activity is essential for certain memory processes (Silva, 1992). We found that autophosphorylation of α CaMKII was significantly stimulated by 10 min of E2 and not vehicle treatment, and this increase was blocked by KN-62 pre-treatment in the primary hippocampal neurons. Clearly, rapid E2 action allows for the short-term autophosphorylation of α CaMKII in the 2 cell lines examined.

Numerous studies indicate that E2's rapid effects in the brain are initiated outside of the nucleus (Kuroki, 2000; Mannella, 2006). Our ER α localization data in NLT neurons corresponds with other groups in that "classical" ERs are positioned not only in the nucleus, but in the cytoplasm and outgrowths as well (Clarke, 2000; Hart, 2007) where they are available to interact with cytoplasmic signaling cascades. Immunofluorescence and cell fractionation showed that E2-induced α CaMKII autophosphorylation in NLT neurons occurs specifically in the cell body and processes, suggesting that the extranuclear pool of ER is responsible for mediating this event. ER action is clearly required as ICI treatment significantly blocks E2-induced α CaMKII autophosphorylation.

Based on our data, ER α and not ER β mediates E2-induced α CaMKII autophosphorylation in both NLT cells and co-transfected Cos7 cells. Use of the subtype-selective compounds, PPT and DPN, in NLT cells as well as the examination of SK-N-SH neuroblastoma cells (ER α -

negative/ER β -positive cells) allowed us to differentiate the effects of ER α versus ER β , and show that ER α is responsible for the α CaMKII autophosphorylation induced by E2 in these particular cellular contexts. Additionally, the transient expression of ER β with α CaMKII in Cos7 cells is unable to mediate kinase autophosphorylation while the co-expression of ER α allows for α CaMKII activity by E2. This result is somewhat surprising because both ER subtypes have been shown to be involved in the rapid induction of Ca²⁺ oscillations in hippocampal neurons (Zhao, 2007), which would presumably result in α CaMKII autophosphorylation mediated by either ER α or ER β . However, closer examination of the hormone-independent association of ER β with α CaMKII may provide insight as to why ER β is unable to elicit kinase autophosphorylation; the ER binding site on α CaMKII overlaps with the CaM binding site so it is possible that ER β competes with the binding of Ca²⁺/CaM and perhaps locks the kinase in an autoinhibited state, thus blocking autophosphorylation by E2. It would be interesting to see if a knock-down ER β in NLT cells or a mutant of ER β that cannot interact with α CaMKII would further enhance α CaMKII autophosphorylation by E2. Nonetheless, our finding that ER α is the sole subtype responsible for α CaMKII autophosphorylation supports the notion that ER α and ER β do not have overlapping roles in brain function. Each subtype has a distinct spatial and temporal expression pattern in the forebrain (Gonzalez, 2007) and they play distinct roles in neuronal function. For example, ER α mediates the neuroprotective effects of E2 against ischemic brain injury (Dubal, 2001) whereas ER β is responsible for the anti-anxiety and anti-depressive effects of E2 (Walf, 2007). However, it has been reported that ER β is involved in CA1 LTP and hippocampal-dependent contextual fear conditioning (Day, 2005), so a closer examination of ER β -selective agonists in the hippocampus is needed to fully appreciate the role of ER β in the activation of α CaMKII within this brain region.

An understanding of the mechanism underlying E2-induced α CaMKII activity is of great interest, and our data indicates that Ca²⁺ influx via L-type Ca²⁺ channels and CaM action are required for α CaMKII autophosphorylation by E2 in both NLT and primary hippocampal neurons. The effects of E2 on Ca²⁺ are well documented; E2 stimulates Ca²⁺ influx (Wu, 2005; Zhao, 2005) as well as the release of Ca²⁺ from intracellular stores (Beyer, 1998). However, the hormone-dependent association of ER α and α CaMKII adds a level of complexity to the signaling. The hypothesis that the ER α - α CaMKII interaction directly activates α CaMKII was proven incorrect and instead, the interaction decreases the ability of E2 to activate α CaMKII by half. Thus, ER α is required initially for the E2-induced α CaMKII activity via Ca²⁺ influx, while it simultaneously downregulates it. As discussed above with ER β , it is possible that the association of ER α with α CaMKII locks the kinase in an autoinhibited state however that hypothesis remains to be tested. These findings are in alignment with studies that examined a transgenic mouse model in which a constitutively active α CaMKII, CaMKII-Asp²⁸⁶, is expressed in an inducible and forebrain-specific fashion (Mayford, 1996; Bejar, 2002; Yasuda, 2006). They found that indiscriminate CaMKII activity results in the loss of low-frequency-induced LTP and deficits in spatial memory. Bejar and colleagues (2002) found that high expression of the transgene also affected fear-conditioned memory. Therefore, it is reasonable to imagine that ER α plays both a positive and negative role in E2-induced α CaMKII activation to prevent molecular and behavioral memory impairments. An alternative explanation is that the opposing effects of ER are in place to “prime” α CaMKII; it prevents α CaMKII from ever reaching maximal activity by E2 stimulation, and instead lowers the threshold for subsequent Ca²⁺ signals, allowing for a more efficient and perhaps, prolonged response. By maintaining sub-maximal activity, the dual role of ER α permits α CaMKII to function as a Ca²⁺ frequency detector to translate the information encoded by the Ca²⁺ spikes into various levels of kinase activity that correspond with specific cellular responses. However, there exists an equally legitimate hypothesis for why the ER α interaction mutant (ER α -V376R) significantly enhances E2-induced α CaMKII autophosphorylation compared to wild-type ER α : perhaps activated ER α -V376R increases other stimulatory actions in the cell such as

Ca^{2+} mobilization to a greater degree than wild-type $\text{ER}\alpha$, which would lead to enhanced αCaMKII autophosphorylation overall. We have not sufficiently tested this theory so it must be considered as a possibility. Still, it is enticing to hypothesize instead that the association of $\text{ER}\alpha$ with αCaMKII occurs to maintain the sensitivity and selectivity of αCaMKII activity in neurons.

It is known that αCaMKII phosphorylates a wide variety of substrates involved in numerous neuronal processes (McGlade-McCulloh, 1993; Omkumar, 1996; Stefani, 1997; Liu, 2005) and we have identified ERK1/2, CREB, and MAP2 as proteins targeted by E2-induced αCaMKII autophosphorylation in NLT and primary hippocampal neurons. Like αCaMKII , all three proteins are involved in neuronal differentiation, and ERK1/2 and CREB have also been shown to play a role in LTP and memory processes (Trifilieff, 2006). E2 action has previously been linked to ERK1/2 and CREB phosphorylation in various populations of neurons and *in vivo* (Lee, 2004; Bryant DN, 2005; Szego, 2006), and we have provided evidence that αCaMKII action mediates their phosphorylation by E2 in NLT and primary hippocampal neurons. CREB can be directly phosphorylated by αCaMKII (Wu, 2001), although in our studies it appears that CREB is indirectly affected by αCaMKII -mediated ERK1/2 activity since its phosphorylation by E2 was blocked by U0126 pre-treatment. This finding corresponds with reports demonstrating that CREB is phosphorylated by ribosomal S6 kinase (RSK) family members, which are first activated by ERK1/2 (Cammarota, 2001). To the best of our knowledge we are the first to report that E2 is capable of stimulating MAP2 phosphorylation in primary hippocampal neurons. MAP2 is a direct target of αCaMKII , although it can also be phosphorylated by ERK1/2 (Sanchez, 2000) as we have confirmed in our studies; its phosphorylation by E2 is blocked by U0126 pre-treatment. Expectedly, both Ca^{2+} -influx and CaM signaling are required for E2-induced ERK1/2, CREB, and MAP2 phosphorylation that is mediated by αCaMKII .

The use of pharmacological inhibitors and RNAi technology allowed us to order the observed E2-induced signaling (Fig. 9). $\text{ER}\alpha$ is rapidly activated by E2, which elevates intracellular Ca^{2+} levels by Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels. αCaMKII detects the Ca^{2+} spike and undergoes autophosphorylation to become transiently active and mediate ERK1/2 activation. ERK1/2 subsequently leads to the phosphorylation of CREB and MAP2. However, the interaction of $\text{ER}\alpha$ with αCaMKII decreases E2-induced kinase autophosphorylation even though ER is initially required for this event via Ca^{2+} influx. It appears as though the activating signal of E2 dominates the negative effect of ER since there is a clear, positive downstream response to E2-activated αCaMKII .

In summary, E2 rapidly evokes the $\text{ER}\alpha$ -dependent autophosphorylation of αCaMKII in co-transfected Cos7 cells, immortalized GnRH neurons, and primary hippocampal neurons. The activity requires Ca^{2+} influx and CaM action, and is kept in check by the interaction of $\text{ER}\alpha$ with αCaMKII , which potentially maintains the sensitivity and selectivity of αCaMKII activity. Ultimately, E2-stimulated αCaMKII activity results in the phosphorylation of ERK1/2, CREB, and MAP2, proteins that are all involved in various aspects of multiple neuronal processes, including proliferation, differentiation, and LTP. The current study increases our understanding of the mechanistic contribution of rapid E2 signaling in the brain, which is necessary for the development of hormonal therapies that specifically target brain function without increasing the risk of breast and uterine cancer in postmenopausal women.

Experimental Procedures

Chemicals and Antibodies

17 β -estradiol (E2), trans-4-hydroxytamoxifen (T), KN-62, BAPTA-AM, Nifedipine, W7, U0126, forskolin, and A23187 were purchased from Sigma (St. Louis, MO). Propyl pyrazole

triol (PPT), diethylpropionitrile (DPN), ICI 182, 780 (ICI), and calmidazolium were purchased from Tocris Cookson (Ballwin, MO). Anti- α CaMKII used for immunofluorescence, antiphospho- α CaMKII (T286) used for western blotting, anti-histone H1 and anti-ER α (MC-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho- α CaMKII used for IHC, anti-phospho-ERK1/2, anti-phospho-CREB, anti-phospho-MAP2, anti-phospho-ERK5, and anti-phospho-ELK1 were purchased from Cell Signaling (Danvers, MA). Anti- α CaMKII used for WB was obtained from Zymed (Invitrogen). Anti-pan actin was obtained from Chemicon (Billerica, MA) and anti-ER β was purchased from Affinity BioReagents (Golden, CO). Anti-FLAG is from Sigma and the ER α antibody, H222.2, is produced in our lab.

Cell Culture

NLT (mouse gonadotropin releasing hormone (GnRH) immortalized neurons) and Cos7 (monkey kidney) cells were routinely cultured in phenol red-free DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS). SK-N-SH (human neuroblastoma) cells were obtained from the American Type Culture Collection (ATCC) and grown in EMEM (Cambrex) supplemented with 10% FBS. All cells were maintained at 37 C in a 5% CO₂ atmosphere. Primary hippocampal neurons were prepared and provided by the Center for Mental Retardation and Disability (University of Chicago) and maintained in Neurobasal Media A (Invitrogen) supplemented with B27, L-glutamine, and penicillin/streptomycin at 37 C in a 5% CO₂ atmosphere.

Plasmids and Mutagenesis

The wild-type human α CaMKII expression vector, SR α - α CaMKII, was kindly provided by Dr. Howard Schulman (Stanford University, CA). The wild-type human ER α (HEGO) is expressed in the pSG5 vector and was a gift from Dr. Pierre Chambon (Institute de Chimie Biologique, France). pcDNA3.1- α CaMKII was obtained by subcloning SR α - α CaMKII into the pcDNA3.1(-) vector (Invitrogen). FLAG-tagged ER α and ER β were obtained by subcloning the receptor into pCMV-FLAG expression vector (Sigma). Site-directed mutagenesis was performed to create α CaMKII-L304A and ER α -V376R with the QuickChange Site-Directed Mutagenesis kit (Stratagene, CA) according to the manufacturer's instructions.

Transient Transfections and Luciferase Assays

For transfections, Cos7 cells were seeded in DMEM+10% FBS in 100mm dishes for co-immunoprecipitation (CoIP) and in 6-well dishes for western blot analysis. For CoIP, Polyfect (QIAGEN) was used to transfect Cos7 cells with either wild-type ER α (pSG5-HEGO or FLAG-ER α , depending on the experiment), FLAG-ER β , or HEGO-V376R and pcDNA3.1- α CaMKII in DMEM+10% dextran-coated charcoal stripped fetal bovine serum (S-FBS). 48 hr posttransfection, the cells were used in CoIP studies. For rapid ligand treatments, cells were transfected with Polyfect in DMEM+10% S-FBS with wild-type pcDNA3.1- α CaMKII in the presence or absence of the following ER constructs: FLAG-ER α , FLAG-ER β , pSG5-HEGO, or HEGO-V376R. 48 hr post-transfection, treatments were reconstituted in PBS, ethanol or DMSO and cells were treated with the concentrations and times indicated. For luciferase assays, NLT cells were seeded in DMEM+10% FBS in 48-well dishes and transfected Lipofectamine (Invitrogen) according to the manufacturer's instructions in DMEM + 10% S-FBS with 3ERE-Luc (estrogen response element-luciferase) reporter and β -galactosidase (β -Gal) expression plasmid or control vector. Additionally, SK-N-SH cells were seeded in EMEM +10% FBS and transfected with Polyfect in DMEM+10% S-FBS with 3ERE-Luc and β -Gal. The day after transfection, treatments were administered to the cells at the dilution indicated in fresh DMEM+10% S-FBS. The next day, cells were harvested and assayed for both

luciferase and β -Gal activity (Microbeta Wallac, Perkin Elmer; Spectra Max 250, Molecular Devices). Luciferase activity was normalized for transfection efficiency using β -Gal and reported as a fold-induction over control.

Cellular Fractionation

Nuclear and cytosolic proteins were extracted as previously described (Sun, 2001). After treatment, NLT cells were washed with ice-cold PBS containing phosphatase inhibitors. The cells were extracted in TNM buffer (10mM Tris, pH 7.4, 100mM NaCl, 2mM MgCl_2 , 300mM sucrose, 1% thiodiethanol, 1mM Na_3VO_4 , 50mM NaF, 1:1000 PIC), disrupted with a syringe and needle, and centrifuged at $4500 \times g$ for 10 min at 4C. The remaining samples were centrifuged at $4500 \times g$ for 10 min at 4C, and the resulting supernatant was retained as the cytosolic fraction. The pellet (crude nuclear fraction) was resuspended in an equal volume of TNM buffer. Whole cell, cytosolic fraction, and nuclear fraction samples were analyzed for the indicated proteins by western blot analysis.

Western Blot Analysis

For western blotting, cells were washed with ice-cold PBS containing phosphatase inhibitors, lysed in 2x laemelli sample buffer, and boiled for 5 min. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and detected by immunoblotting with the indicated primary antibodies and fluorescently-tagged secondary antibodies (Molecular Probes, Invitrogen) using the Odyssey Infrared System (Li-COR, NE). Both phosphorylated and total protein expression were quantified by densitometry using the Odyssey software (Li-COR). Phosphorylated levels of αCaMKII , ERK1/2, and CREB were all normalized to total protein levels, and to avoid inter-assay variations, the values obtained were normalized with the value measured for the non-treated control samples (C) in each experiment. Data are presented as the mean fold increase $\pm$ S.D. for at least 3 independent experiments. Student's t-test was used to produce P values.

Immunofluorescence

For immunofluorescence, NLT cells were plated in a 96-well black/clear bottom dish in DMEM with 10% FBS for 24 hr, then grown for an additional 16hr in DMEM with 10% S-FBS. Cells were treated as indicated, fixed in buffered formalin (Fisher Scientific) for 15 min, and then ice-cold methanol was applied for 10 min. The cells were blocked in 5% goat serum in PBS/0.3% Triton X-100 and stained overnight at 4C with both anti-ER α (1:100, Santa Cruz Biotechnology) and anti- αCaMKII (1:100, Santa Cruz Biotechnology) for colocalization, or anti-phospho- αCaMKII (1:200, Santa Cruz Biotechnology) alone to detect kinase activation. Alexa 594-conjugated goat anti-rabbit and Alex 488-conjugated goat anti-mouse (1:2000 and 1:2500 respectively, Molecular Probes) were applied for 1 hr, followed by DAPI staining (Molecular Probes). Images were acquired using the IXMicro imager and analyzed with MetaXpress software (Molecular Devices, CA).

GST Pull-Down Assay

pcDNA3.1- αCaMKII constructs were radiolabeled with ^{35}S -Methionine (Amersham Biosciences) using the TnT Quick Coupled Transcription/Translation System by Promega Corp. (Madison, WI) following the manufacturer's instructions exactly. The resulting labeled proteins were incubated with either wild-type GST-ER α -LBD or one of the following GST-LBD mutants, GST-ER α -I358R, GST-ER α -V376R, or GST-ER α -E542K bound to glutathione-sepharose beads for 1.5 hr at 4C in the presence of the ligand indicated. Samples were washed 3 times with binding buffer (20mM Tris, pH 7.6; 50mM NaCl; 0.05% Nonidet P-40; 1mM dithiothreitol; 1:1000 protease inhibitor cocktail) and bound proteins were eluted

with 2x laemmli sample buffer. After 5 min boiling, the samples were separated by SDS-PAGE and analyzed by autoradiography on the STORM scanner (GE Healthcare BioSciences).

Colmunoprecipitation

Cos7 cells were plated and transfected as described above and then treated with either vehicle (0.01% EtOH) or E2 (10nM) for 10 min, washed with ice-cold PBS and extracted in lysis buffer (50mM Tris, pH 8.0, 150mM NaCl, 10mM MgCl₂, 1% NP-40, 1mM Na₃VO₄, 50mM NaF, 1:1000 PIC). The resulting cell lysates were incubated for 1 hr with Protein A/G-PLUS Agarose beads (Santa Cruz Biotechnology, CA) at 4C to preclear the lysates. Depending on the experiment ER α was subsequently immunoprecipitated with either 2 μ g anti-FLAG or 2 μ g H222.2, ER β was immunoprecipitated with 2 μ g anti-FLAG, and α CaMKII was immunoprecipitated with 4 μ g of anti- α CaMKII in the presence of Protein A/G PLUS agarose overnight at 4 C. The samples were gently washed 3 times in wash buffer (20mM Tris, pH 7.6, 50mM NaCl, 0.05% Nonidet P-40, 1mM dithiothreitol, 1:1000 PIC) and bound proteins were eluted with 2x laemmli sample buffer. Samples were boiled and western blotting was performed to detect ER α , ER β , and α CaMKII.

siRNA Silencing Experiments

Primary hippocampal neurons were allowed to grow in Neurobasal media supplemented with B27 for 5 days and then the indicated ON-TARGET plus siRNA was introduced using the Dharmafect system according to the manufacturer's instructions (Dharmacon Inc., CO). Briefly, 200nM of α CaMKII or non-targeting siRNA was transfected into neurons using Dharmafect 3. The cells were incubated at 37 C for 60 hr with the siRNA of interest, treated as indicated, and then assayed for the expression levels of the targeted protein via western blot analysis. Additionally, the phosphorylation status of a variety of proteins including α CaMKII, ERK1/2, CREB, and MAP2 was examined post-siRNA and ligand treatment. Total α CaMKII levels were normalized to Actin and presented as the mean relative protein expression $\pm$ S.D. The phosphorylated levels of α CaMKII, ERK1/2, CREB, and MAP2 were normalized to total protein levels, and to avoid inter-assay variations, the values obtained were normalized with the value measured for the non-treated control samples (C) in each experiment. Data are presented as the mean fold increase $\pm$ S.D. and P values were calculated with the Student's t-test.

Abbreviations

ER, estrogen receptor; E2, 17 β -estradiol; ERE, estrogen response element; LBD, ligand-binding domain; T, tamoxifen; PPT, propyl pyrazole triol; DPN, diarylpropionitrile.

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Figure 1. NLT cells express functional ERα and ERβ and E2 rapidly induces the autophosphorylation of αCaMKII

(A) Unstimulated NLT cells were co-stained for ERα (red) and DAPI (blue). Left panel: immunofluorescence performed with primary antibody (MC-20). Right panel: immunofluorescence performed in the absence of primary antibody (B) NLT cells were either unstimulated (C) or treated for 10 min with E2 (10nM), and immunoblotting detected total ERα and pan-Actin. (C) NLT cells were transfected with 3ERE-Luc and β-galactosidase, treated with vehicle (Veh; 0.01% EtOH), E2 (10nM), E2+ICI (10nM, 1uM), PPT (100nM), PPT+ICI (100nM, 1uM), DPN (100nM), or DPN+ICI (100nM, 1uM) for 24 hr, and assayed for both luciferase and β-galactosidase activity. Luciferase activity was normalized for

transfection using β -galactosidase and represented as fold increase RLU (relative light units) over vehicle-treated cells. **(D)** NLT cells were either unstimulated (C) or treated with increasing concentrations of E2 (0.1nM–1000nM) for 10 min, and autophosphorylated α CaMKII and pan-Actin levels were detected by immunoblotting. Representative blot. **(E)** NLT cells were treated with forskolin and A23187 (F/A) for 2 min and 20 min, respectively, as a positive control, or with E2 (10nM) or vehicle (0.01% EtOH) for the indicated times. Immunoblotting detected autophosphorylated α CaMKII and pan-Actin. **(F)** NLT cells were either unstimulated (C) or treated with vehicle (Veh; 0.01% EtOH), E2 (10nM), E2+ICI (E+I; 10nM, 1uM), or E2+KN-62 (E+K; 10nM, 10uM) for 10 min. ICI and KN-62 were applied 30 min prior to E2 treatment. Immunoblotting detected autophosphorylated α CaMKII and pan-Actin. Representative blot. Densitometry for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control $\pm$ S.D. (*) $p < 0.01$, E2 relative to other treatment conditions.

Figure 2. E2-induced α CaMKII autophosphorylation occurs in the cytoplasm and cell extensions of NLT cells in a Ca^{2+} /CaM-dependent manner

(A) Cells were either unstimulated (C) or treated with E2 (10nM) or E2+KN-62 pre-treatment (10nM, 10uM) for 10 min and then stained for autophosphorylated α CaMKII (red) and DAPI (blue) to visualize localization of kinase activation. (B) Whole cell extract (WCE), cytosolic, and nuclear fractions were obtained from NLT cells treated with vehicle (Veh; 0.01% EtOH), F/A (0.5mM, 50uM), E2 (10nM), or E2+KN-62 30 min pre-treatment (10nM, 10uM), and analyzed by immunoblotting for autophosphorylated α CaMKII, GAPDH (cytosolic protein), and Histone H1 (nuclear protein). (C) NLT cells were either unstimulated (C) or treated for 10 min with Veh (0.01% EtOH), F/A (0.5mM, 50uM), E2 (10nM), or E2 (10nM) with 30 min pre-

treatment of KN-62 (E+K; 10uM), Nifedipine (E+Nif; 10uM), BAPTA-AM (E+B; 10uM), W7 (E+W; 10uM), or ICI (E+I; 1uM). Representative blot. **(D)** Autophosphorylated α CaMKII was normalized to total protein levels from 3 independent experiments and presented as the mean fold increase over unstimulated control $\pm$ S.D. (*) $p < 0.03$, E2 relative to other treatment conditions.

Figure 3. ERα and not ERβ mediates αCaMKII autophosphorylation

(A) NLT cells were either unstimulated (C) or treated with Veh (0.01% EtOH) or increasing concentrations of PPT or DPN (1nM–1000nM) for 10 min. Immunoblotting was used to detect autophosphorylated αCaMKII, ERα, ERβ, and pan-Actin levels. Representative blot. (B) Densitometry analysis for phosphorylated kinase was normalized to total protein levels from 3 independent experiments, and presented as the mean fold increase over unstimulated control ± S.D. (*) $p < 0.02$. (C) SK-N-SH cells were treated with Veh (0.01% EtOH), F/A (0.5mM, 50uM), or E2 (10nM) for the times indicated and immunoblotting was used to detect total and autophosphorylated αCaMKII. (D) SK-N-SH cells were transfected with 3ERE-Luc and β-galactosidase, treated with Veh (0.01% EtOH), E2 (10nM), E2+ICI (10nM, 1uM), PPT

(100nM), DPN (100nM), or ICI (1uM) for 24 hours, and assayed for both luciferase and β -galactosidase activity. Luciferase activity was normalized for transfection using β -galactosidase and represented as fold increase RLU over vehicle-treated cells. **(E)** Cos7 cells co-transfected with α CaMKII and either ER α or ER β were unstimulated (C) or treated with Veh (0.01% EtOH), F/A (0.5mM, 50uM), E2 (10nM), or E2+KN-62 pre-treatment (E+K; 10nM, 10uM) for 2 min and then subjected to immunoblotting to detect autophosphorylated α CaMKII, total α CaMKII, FLAG-tagged ER α or ER β , and pan Actin. Representative blot. **(F)** Densitometry analysis for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control $\pm$ S.D. (*) $p < 0.02$.

Figure 4. ERα interacts with αCaMKII in a hormone-dependent manner to attenuate E2-induced αCaMKII activity

(A) GST-ERα-LBD pulldown of [³⁵S]-αCaMKII (wild-type and L304A). See materials and methods for details. V = vehicle, 1% EtOH; E = E2, 1μM; T = 4-hydroxy-tamoxifen, 1μM. Bound αCaMKII was visualized by autoradiography. (B) GST-ERα-LBD pulldown of wild-type [³⁵S]-αCaMKII using wild type GST-ERα-LBD or GST-ERα- I358R, GST-ERα-V376R, or GST-ERα-E542K. V = vehicle, 1% EtOH; E = E2, 1μM. Bound αCaMKII was visualized by autoradiography. (C) Cos7 cells were co-transfected with FLAG-ERα and αCaMKII, treated with Veh (0.01%EtOH) or E2 (10nM) for 10 min, and ERα and αCaMKII were immunoprecipitated with either anti-FLAG or anti-αCaMKII, respectively. Immunoblotting

was used to detect ER α and α CaMKII. **(D)** Cos7 cells were co-transfected with FLAG-ER β and α CaMKII, treated with Veh (0.01% EtOH) or E2 (10nM) for 10 min, and ER β and α CaMKII were immunoprecipitated with either anti-FLAG or anti- α CaMKII, respectively. Immunoblotting was used to detect ER β and α CaMKII. **(E)** Cos7 cells were co-transfected with α CaMKII and either wild type ER α or the interaction mutant ER α (V376R), treated with Veh (0.01% EtOH) or E2 (10nM) for 10 min, and ER α was immunoprecipitated with H222.2. Immunoblotting was used to detect ER α and α CaMKII. **(F)** Cos7 cells were co-transfected with α CaMKII and either wild-type ER α or the interaction mutant ER α (V376R), treated with F/A (0.5mM, 50uM), E2 (10nM), E2+BAPTA-AM pre-treatment (E+B; 10nM, 10uM) for 2 min, or left unstimulated (C). Immunoblotting detected autophosphorylated α CaMKII, total α CaMKII, ER α , and pan-Actin. Representative blot. **(G)** Density values for autophosphorylated α CaMKII were normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control $\pm$ S.D. (*) $p < 0.03$, (**) $p < 0.02$.

A.

B.

C.

D.

Figure 5. α CaMKII and Ca^{2+} /CaM signaling are involved in E2-induced ERK1/2 and CREB phosphorylation

(A) NLT cells were either unstimulated (C) or treated for 10 min with Veh (0.01% EtOH), E2 (10nM), or E2 (10nM) with 30 min pre-treatment of KN-62 (E+K; 10uM), ICI (E+I; 1uM), Nifedipine (E+N; 10uM), or U0126 (E+U; 10uM). Immunoblotting detected phosphorylated levels of ERK1/2 and CREB as well as pan-Actin. Representative blot. (B) Densitometry analysis for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control $\pm$ S.D. (*) $p < 0.03$, E2 compared to all other conditions. (C) NLT cells were either unstimulated (C) or treated for 10 min with Veh (0.01% EtOH), E2 (10nM), or E2 (10nM) with 30 min pretreatment

of calmidazolium (E+Calm; 10uM). Immunoblotting detected phosphorylated ERK1/2 and CREB, and pan-Actin. Representative blot. **(D)** Densitometry for phosphorylated kinase was normalized to total protein levels, and presented as the mean fold increase over unstimulated control $\pm$ S.D. (*) $p < 0.02$.

A.

B.

Figure 6. Rapid E2 action induces α CaMKII autophosphorylation as well as the phosphorylation of downstream proteins in a α CaMKII-dependent manner in primary hippocampal neurons

(A) Cultured embryonic primary hippocampal neurons were either unstimulated (C) or treated with Veh (0.01% EtOH), E2 (1nM), or E2 (1nM) with 30 min pretreatment of KN-62 (E+K; 10uM), ICI (E+I; 1uM), or U0126 (E+U; 10uM). Immunoblotting detected phosphorylated levels of α CaMKII, ERK1/2, ERK5, ELK1, CREB, and MAP2 as well as pan-Actin. Representative blot. (B) Densitometry for phosphorylated kinase was normalized to total protein levels for 3 independent experiments, and presented as the mean fold increase over unstimulated control $\pm$ S.D. a) $p < 0.02$; b) $p < 0.05$; c) $p < 0.04$; d) $p < 0.05$, comparing E2 treatment to all other conditions.

A.

B.

Figure 7. Ca^{2+} /CaM action are required for E2-induced ERK1/2, CREB, and MAP phosphorylation in primary hippocampal neurons

(A) Cultured embryonic primary hippocampal neurons were either unstimulated (C) or treated with Veh (0.01% EtOH), E2 (1nM), or E2 (1nM) with 30 min pre-treatment of BAPTA-AM (E+B; 10uM) or calmidazolium (E+Calm; 10uM). Immunoblotting detected phosphorylated levels of αCaMKII, ERK1/2, CREB, and MAP2 as well as pan-Actin. Representative blot. (B) Densitometry for phosphorylated kinase was normalized to total protein levels, and presented as the mean fold increase over unstimulated control ± S.D. (*) p < 0.01, (**) p < 0.02.

Figure 8. Targeted inhibition of αCaMKII blocks E2-induced ERK1/2, CREB, and MAP2 phosphorylation

(A) Cultured embryonic primary hippocampal neurons were transfected with either non-targeting siRNA (siN.T.) or siαCaMKII, and were unstimulated (C) or treated with Veh (0.01% EtOH) or E2 (1nM). Total αCaMKII, autophosphorylated αCaMKII, phosphorylated levels of ERK1/2, CREB, MAP2, and pan-Actin expression were then assayed via immunoblotting. Representative blot. (B) Densitometry for total αCaMKII was normalized to pan-Actin and presented as the mean relative protein expression ± S.D. Additionally, the phosphorylation status of αCaMKII (C), ERK1/2 (D), CREB (E), and MAP2 (F) was examined post-siRNA introduction and ligand treatment. Phosphorylated levels of the indicated proteins were

normalized to pan-Actin and presented as the mean fold increase over unstimulated control $\pm$ S.D. * $p < 0.01$; ** $p < 0.03$; *** $p < 0.05$.

Figure 9. Model of E2-induced α CaMKII signaling

E2 activates ER α , elevating intracellular Ca²⁺ levels by inducing Ca²⁺ influx via L-type Ca²⁺ channels. The Ca²⁺ spike is detected by α CaMKII, which indirectly phosphorylates ERK1/2 after being activated. Active ERK1/2 then results in the phosphorylation of CREB and MAP2. The ER α - α CaMKII interaction attenuates the ability of E2 to stimulate kinase autophosphorylation even though the receptor is initially required for E2 to induce α CaMKII activity. However, it appears as though the activating signal of E2 (thick arrows) supercedes the negative effect of ER since there is a clear, positive downstream response to E2-activated α CaMKII.