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14. ABSTRACT: Recent evidence has made it clear that ER-mediated extranuclear signaling is involved in the growth and survival of ER-expressing cells and tissues, including both reproductive and nonreproductive. Specifically, we are interested in examining the ability of ER action to rapidly modulate various signaling cascades, and our main goals with this research are to: 1) define the mechanism responsible for the rapid ER signaling, 2) investigate the observed signaling in an animal model, 3) determine and compare the target genes that are regulated by ER rapid signaling versus classical ER transactivation, and 4) examine the subsequent cellular and biological responses to rapid 17 β -estradiol (E2) action. Previously, we confirmed that E2 and other ER-specific ligands can rapidly phosphorylate and activate Erk1/2 in the breast cancer cell line, MCF-7, an effect that is blocked by the potent ER antagonist, ICI 182, 780. We have also provided evidence that E2 administration to ovariectomized immature rats can induce Erk1/2 phosphorylation in the uterine horn and brain and that E2 can also induce α CaMKII autophosphorylation in the brain in vivo. α CaMKII was also identified as an upstream regulator of E2-induced ERK1/2 phosphorylation. We show here that E2 can rapidly and significantly induce α CaMKII autophosphorylation, which subsequently mediates ERK1/2 phosphorylation in immortalized GnRH neurons and primary hippocampal cells. This signaling results in p90RSK and CREB phosphorylation that appears to require Ca ²⁺ influx through the L-type Ca ²⁺ channels. Interestingly, the administration of either E2 or PPT, the ER α -selective agonist, but not vehicle, to female ovariectomized rats results in a clear enhancement of α CaMKII autophosphorylation in the hippocampus. Our findings suggest a novel model for the activation of ERK1/2 by ER α via α CaMKII signaling, which increases our understanding of E2 action in the central nervous system. Ultimately, the characterization of this signaling pathway could be exploited to create new selective estrogen receptor modulators (SERMs) that can potentially enhance memory and cognitive function in postmenopausal women without affecting breast tissue or increasing the risk of developing breast cancer.					
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Introduction:

Recent evidence has made it clear that ER-mediated extranuclear signaling is involved in the growth and survival of ER-expressing cells and tissues, including both reproductive and nonreproductive (1, 2). We have pursued this research in order to gain a better understanding of rapid estrogen action on targeted signaling cascades, including the MAPK and Ca^{2+} /Calmodulin-dependent kinase II (CaMKII) pathways. CaMKII is a multi-subunit kinase that is exquisitely responsive to Ca^{2+} levels and consists of 4 distinct isoforms, α -, β -, δ -, and γ CaMKII. δ - and γ CaMKII have a relatively ubiquitous expression pattern while α - and β CaMKII are prevalent in brain tissue; in fact, α CaMKII comprises approximately 2% of total protein in the rat forebrain (3). α CaMKII is essential for the induction of long-term potentiation (4-6), which is thought to be the molecular mechanism underlying learning and memory. The goals of this project are to better define the mechanism responsible for ER-mediated ERK1/2 signaling and examine it in an animal model. In order to gain insight into the downstream effects of ER-mediated extranuclear signaling, we will determine and compare the target genes that are regulated by ER rapid signaling versus classical ER transactivation, and examine the subsequent cellular and biological responses.

Body:

Previously, we confirmed that 17 β -estradiol (E2), diethylstilbestrol (DES), propyl pyrazole triol (PPT), and 4-estren-3 α -17 β -diol (Estren) can rapidly phosphorylate and activate ERK1/2 in the breast cancer cell line, MCF-7, which is effectively blocked by the potent ER antagonist, ICI 182, 780 (ICI). Using the ovariectomized female rat as an animal model, we demonstrated that E2 can elicit ERK1/2 phosphorylation *in vivo* in both the uterine horn and brain after intraperitoneal (I.P.) injection and that E2 administration can also induce α CaMKII autophosphorylation in the brain. We identified α CaMKII as an upstream regulator of rapid E2-induced ERK1/2 phosphorylation in immortalized NLT GnRH neurons, which gave us our first glimpse of a novel mechanism by which ER can signal to ERK1/2. Importantly, we also showed that E2 can rapidly induce the autophosphorylation and activation of α CaMKII itself.

We continued our investigation of ER-mediated ERK1/2 phosphorylation with a closer examination of CaMKII signaling. CaMKII is a ubiquitous kinase that a former graduate student in the lab previously demonstrated to interact with ER α in breast cancer cells. She also showed that CaMKII can phosphorylate ER in the ligand-binding domain, a modification that enhances the receptor's ability to function as a transcription factor in breast cancer cells. As we continued to obtain inconsistent data with MCF-7 cells, we focused on the NLT GnRH neurons as a cellular model. We found that these cells express both ER α and α CaMKII by western blot analysis (data not shown), and immunofluorescence showed that the two proteins colocalize in the cytoplasm (Fig. 1A). Treatment with E2 but not vehicle results in a peak of α CaMKII autophosphorylation by 10 min, and this increase is significantly blocked by both KN-62, a CaMKII-specific inhibitor, and ICI (Fig. 1B). E2-induced α CaMKII autophosphorylation occurs predominantly in the cytoplasm of the cells as indicated by both immunofluorescence (Fig. 1C) and cellular fractionation (Fig. 1D).

We next wanted to determine if E2-stimulated α CaMKII autophosphorylation is mediated by ER α only, as we found with an *in vitro* kinase assay (data not shown), or if ER β is also capable of kinase autophosphorylation. To address this question, we first examined the effect of two ER-selective ligands, PPT, an ER α -selective agonist, and DPN, an ER β -selective agonist. Western blot analysis demonstrated that while PPT is able to rapidly induce α CaMKII autophosphorylation in a dose-dependent manner, DPN is unable to elicit the same response even at the highest dose (Fig. 2A). Additionally, we found that E2 treatment is unable to induce α CaMKII autophosphorylation at any of the time points examined in SK-N-SH cells, which is a neuroblastoma cell line that we have found to be void of ER α but contains functional ER β as evidenced by reporter assays (Fig. 2B). Finally, we investigated the effect of rapid E2 action on Cos7 cells that were transfected with α CaMKII alone or in the presence of either FLAG-ER α or FLAG-ER β . The co-expression of ER α but not ER β with α CaMKII results in increased kinase autophosphorylation after just 2 min of E2 but not vehicle treatment. Importantly, α CaMKII expression alone is inadequate for E2-induced kinase activation while it retains the ability to autophosphorylate in response to calcium mobilization stimulated by forskolin and A23187 treatment (Fig. 2C). These data suggest that ER α is the receptor subtype responsible for α CaMKII autophosphorylation induced by E2 treatment of immortalized neuronal and co-transfected Cos7 cells.

We wanted to confirm that E2 can rapidly stimulate ERK1/2 activation via CaMKII signaling, as well as look at the phosphorylation status of downstream proteins, including CREB and p90RSK, which are known targets for ERK1/2 (7). 10 min of E2 treatment to NLT cells results in the phosphorylation of all three proteins in a CaMKII-dependent manner as it is blocked by pre-treatment with KN-62, the specific CaMKII inhibitor. The phosphorylation is also dependent upon Ca^{2+} influx from L-type Ca^{2+} channels as it is inhibited by Nifedipine pretreatment (Fig. 3A). Additionally, both CREB and p90RSK phosphorylation is dependent on ERK1/2 activation as treatment with the MEK inhibitor U0126 30 min prior to E2 significantly reduces the phosphorylation of these two molecules without affecting α CaMKII autophosphorylation (Fig. 3A/B), suggesting that α CaMKII activation is the upstream signaling event.

We continued to investigate E2-induced α CaMKII autophosphorylation *in vivo* and administered vehicle (10% cremaphor/2% EtOH in saline), E2 (0.2ug/rat), or PPT (10ug/rat) subcutaneously to ovariectomized female rats for 15 min, 1 hr, or 24 hr. Immunohistochemistry for autophosphorylated α CaMKII and total CaMKII was performed and we found that both E2 and PPT administration enhances α CaMKII autophosphorylation over unstimulated and vehicle-injected animals in the hippocampus after 1 hr and 24 hr of treatment, whereas only PPT is capable of inducing autophosphorylation at 15 min (Fig. 4A, top panels). Upon closer examination of the hippocampal structures, we found that exposure to PPT for 15 min moderately enhances kinase activity in the CA1, CA2, and CA3 pyramidal neurons while stimulating a dramatic induction of kinase activity in the dentate gyrus (DG) (Fig. 4A, bottom panels). Both E2 and PPT are able to enhance α CaMKII activity by 1 hr of exposure, with the most significant activity occurring in the DG, and PPT again induces kinase activity in the CA1, CA2, and CA3 neurons while E2 has a minimal effect in these areas. However, by 24 hr after E2- α CaMKII autophosphorylation is evident in all hippocampal structures examined, with the most striking effect in the CA3 neurons and DG. PPT continues to

induce α CaMKII autophosphorylation at 24hr in all structures examined. These data suggest that ER α -mediated α CaMKII autophosphorylation does occur *in vivo* and may be involved in a variety of physiological responses, and as the hippocampus is targeted, learning and memory processes may be influenced by this signaling event.

As α CaMKII was targeted for autophosphorylation by E2 and PPT in the hippocampus *in vivo*, we decided to examine the effect of E2 on CaMKII signaling in primary hippocampal neurons. E2 treatment for 10 min results in the autophosphorylation of α CaMKII as well as the phosphorylation of ERK1/2, CREB, and microtubule associated protein 2 (MAP2). The phosphorylation status of ELK-1 and ERK5 is unaltered by E2 treatment. α CaMKII, ERK1/2, CREB, and MAP2 phosphorylation induced by E2 is effectively blocked by KN-62 which suggests that CaMKII is involved in these signaling events. Inhibition of ER with ICI pre-treatment decreased E2-stimulated α CaMKII autophosphorylation as well as ERK1/2, CREB, and MAP2 phosphorylation, which is in agreement with NLT data. Interestingly, blocking MEK1/2 activity with U0126 pre-treatment decreases α CaMKII autophosphorylation as well as ERK1/2, CREB, and MAP2 phosphorylation by E2, suggesting that MEK1/2 can also function upstream of α CaMKII in these cells, which contradicts what was observed in the NLT GnRH neurons in which E2-stimulated α CaMKII autophosphorylation was unaffected by U0126.

The remaining tasks in the statement of work will be addressed and completed in future experiments.

Key Research Accomplishments:

- E2 significantly and rapidly induces α CaMKII autophosphorylation in NLT GnRH neurons and Cos7 cells cotransfected with ER α and α CaMKII.
- ER α and not ER β appears to be responsible for E2-induced α CaMKII autophosphorylation.
- E2-induced α CaMKII autophosphorylation results in ERK1/2 phosphorylation, which subsequently leads to p90RSK and CREB phosphorylation. Ca²⁺ influx is also involved in the phosphorylation of ERK1/2, p90RSK, and CREB.
- *In vivo*, E2 and PPT but not vehicle can enhance α CaMKII autophosphorylation in the hippocampus when administered for 1 hr.
- In primary hippocampal neurons, E2 stimulates the autophosphorylation of α CaMKII as well as the phosphorylation of ERK1/2, CREB, and MAP2 in a CaMKII-dependent manner.

Reportable Outcomes:

Recent Presentations:

Poster Presentation:

O'Neill, E., Blewett, A., Loria, P., Greene, G.

Keystone Symposia, Nuclear Receptors: Steroid Sisters Meeting

Fairmont Banff Springs. Banff, Alberta, Canada – March 2006

O'Neill, E.E., Blewett, A.R., Greene, G.L.
Biomedical Sciences Retreat, University of Chicago
Grand Geneva Resort and Spa. Lake Geneva, WI - May 2006

Upcoming Presentations:

Poster Presentation:

O'Neill, E.E., Blewett, A.R., Greene, G.L.
Biomedical Sciences Retreat, University of Chicago
Lake Lawn Resort. Lake Delavan, WI - April 2007

Oral Presentation:

O'Neill, E.E., Blewett, A.R., Greene, G.L.
The Endocrine Society Meeting
Toronto, Canada – June 2007

Conclusion:

Our findings suggest a novel model for the activation of ERK1/2 by ER α via α CaMKII signaling in immortalized GnRH neurons, primary hippocampal neurons, and the rat hippocampus *in vivo*. We have investigated the relationship between ER and CaMKII signaling in more detail as our previous data demonstrated that it was an important upstream regulator of ERK1/2 phosphorylation by E2. In this report, we have provided evidence that E2-induced α CaMKII activation is most likely mediated by ER α and not ER β and that it ultimately results in the phosphorylation of downstream molecules including ERK1/2, p90RSK, and CREB. Additionally, E2 and PPT can influence α CaMKII autophosphorylation *in vivo* in the rat hippocampus and E2 significantly increases α CaMKII autophosphorylation in primary hippocampal neurons as well as the phosphorylation of ERK1/2, CREB, and MAP2 in a CaMKII-dependent manner.

The outcome of this completed work has several implications. First, it will result in an improved understanding of ER signaling outside of its classical genomic role. Second, it allows for some insight into the mechanisms of E2 action in the brain, which are still not fully understood (8, 9). Finally, examination of ER-mediated extranuclear signaling in the brain has the promise to develop advancements in hormone replacement therapies as it will result in a better understanding of how estrogen functions within the central nervous system. The characterization of E2-induced α CaMKII signaling pathway can be exploited to create new selective estrogen receptor modulators (SERMs) that can potentially enhance memory and cognitive function in postmenopausal women without affecting the breast tissue or increasing the risk of developing breast cancer.

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Appendix 1: Supporting Data

Figure 1

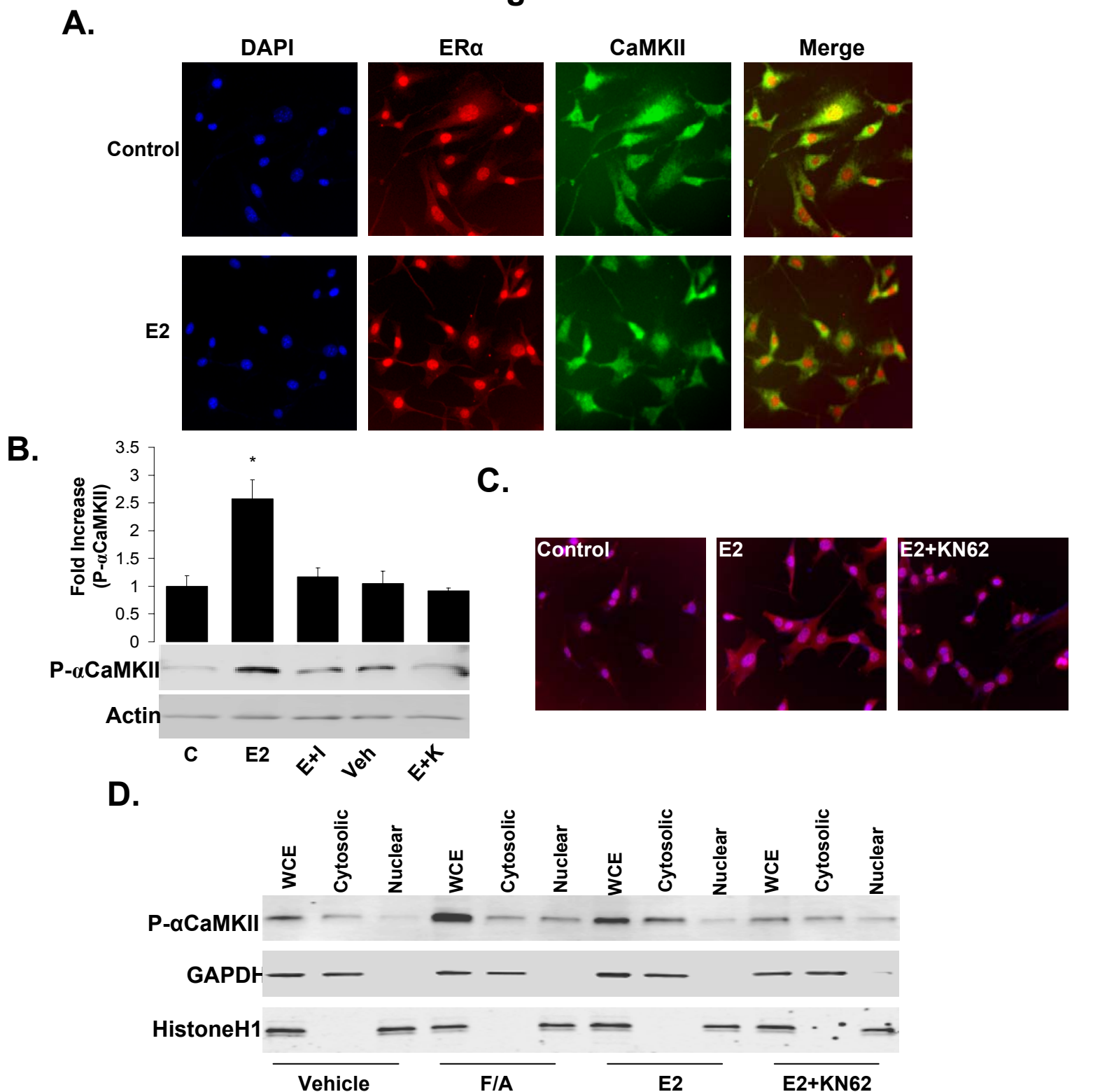


Figure 1. ERα and αCaMKII colocalize and E2 rapidly induces the autophosphorylation of αCaMKII in GnRH neurons, predominantly in the cytoplasmic compartment. a) NLT cells were either unstimulated or treated with 10m of E2 (10nM) and then stained for ERα (red), αCaMKII (green), and DAPI (blue) to visualize localization. b) NLT cells were treated with either vehicle (0.1% EtOH) or E2 (10nM), E2+ICI (10nM, 1uM), or E2+KN62 (10nM, 10uM) for 10 min. The inhibitors, ICI and KN62, were applied 30 min prior to E2 treatment. Immunoblotting was used to detect autophosphorylated αCaMKII and Pan-Actin. Densitometry for phosphorylated kinase was normalized to total protein levels, and presented as the mean fold increase over nonstimulated control ± S.D. for at least 3 independent experiments. c) Cells were either untreated or stimulated with E2 (10nM) or E2+KN62 (10nM, 10uM) for 10 min and then stained for autophosphorylated αCaMKII (red) and DAPI (blue) to visualize localization of kinase activation. d) Whole cell, cytosolic, and nuclear fractions were obtained from NLT cells treated with vehicle, forskolin+A23187 (F/A, 0.5mM, 50uM), E2 (10nM), or E2+KN62 (10nM, 10uM) and analyzed by immunoblotting for autophosphorylated αCaMKII, GAPDH, and Histone H1.

Figure 2

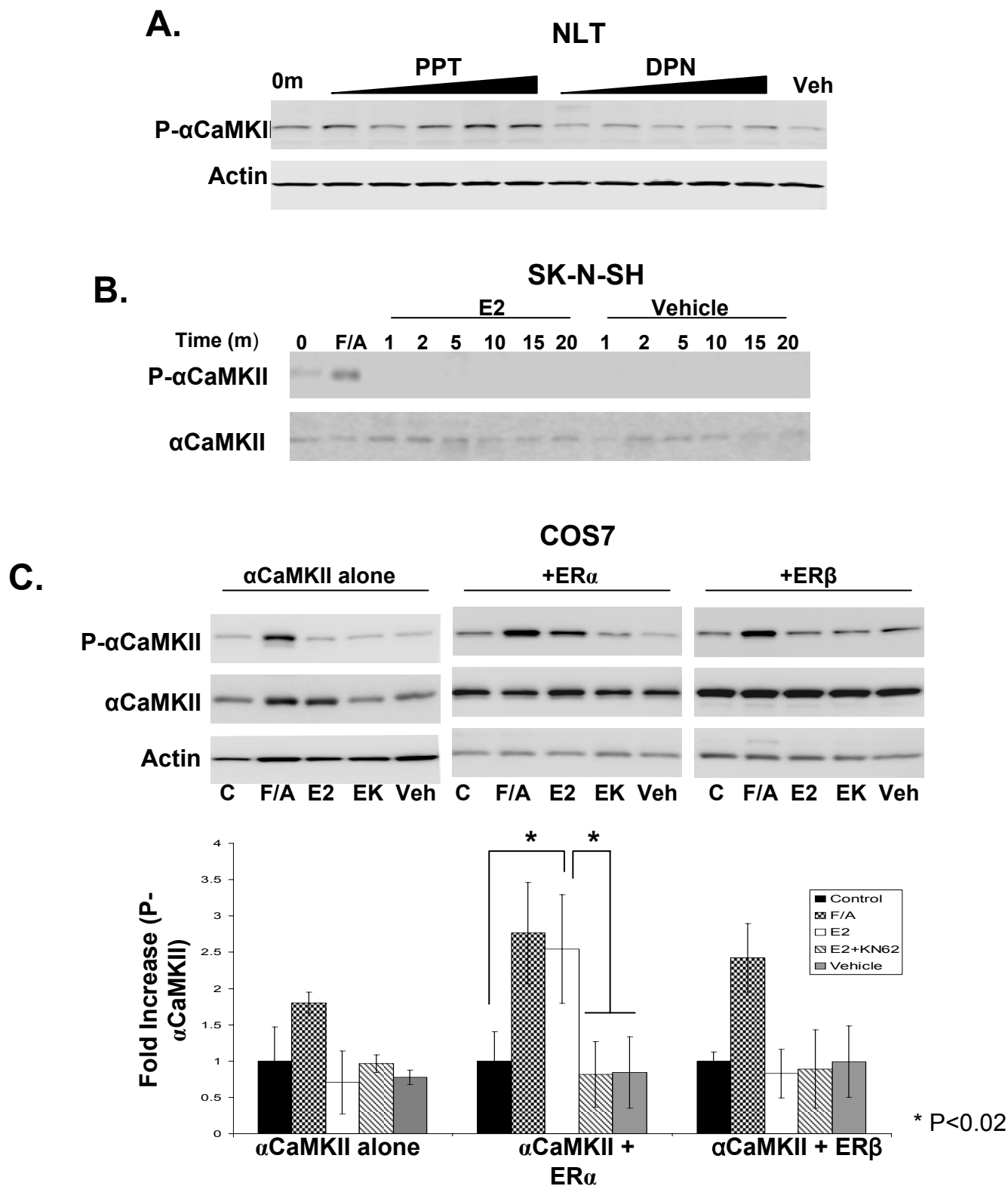
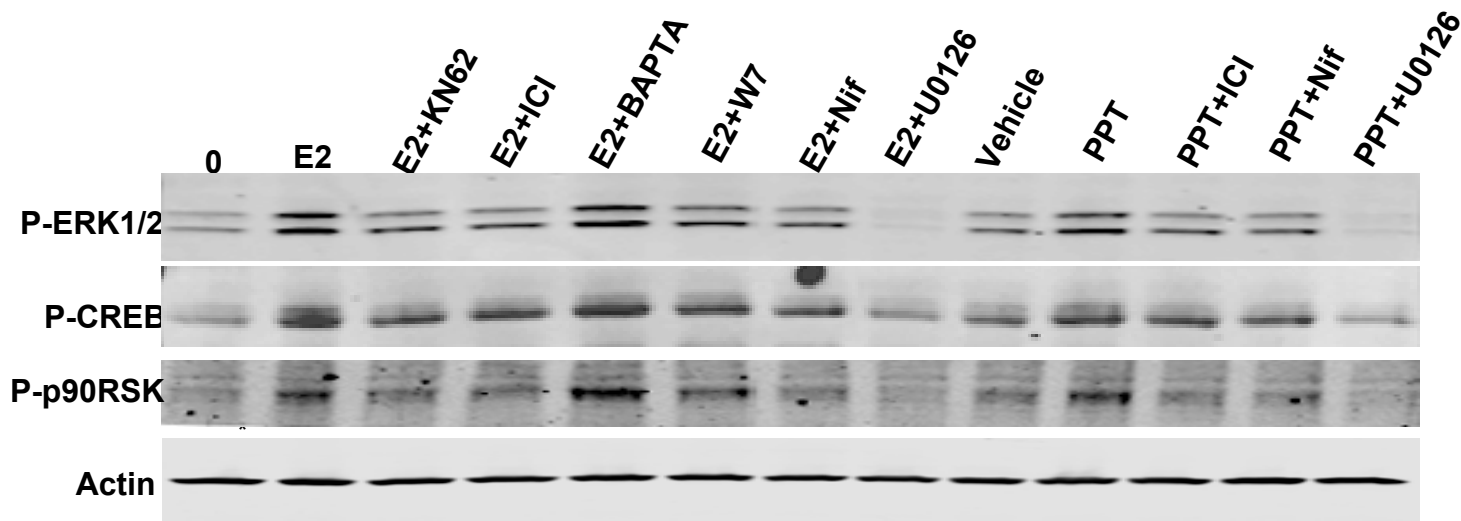


Figure 2. ER α and not ER β mediates α CaMKII autophosphorylation. a) NLT cells were treated with vehicle (0.1% EtOH), increasing concentrations of PPT (1nM-1uM), or increasing concentrations of DPN (1nM-1uM) for 10 min. Immunoblotting was used to detect autophosphorylated α CaMKII and Pan-Actin. b) SK-N-SH cells were treated with either vehicle (0.1% EtOH) or E2 (10nM) for the times indicated and immunoblotting was used to detect autophosphorylated α CaMKII and Pan-Actin. c) Cos7 cells cotransfected with α CaMKII and either ER α or ER β were treated with vehicle (0.1% EtOH), F/A (0.5mM, 50uM), E2 (10nM), or E2+KN62 (10nM, 10uM) for 2 min and then subjected to immunoblotting to detect autophosphorylated α CaMKII, total α CaMKII, and pan Actin. Densitometry for phosphorylated kinase was normalized to total protein levels, and presented as the mean fold increase over nonstimulated control $\pm$ S.D. for at least 3 independent experiments.

Figure 3

A.



B.

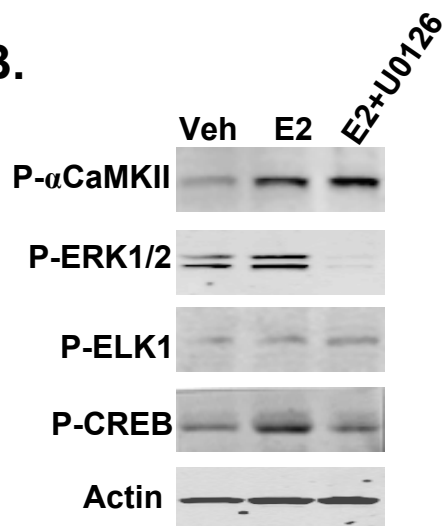
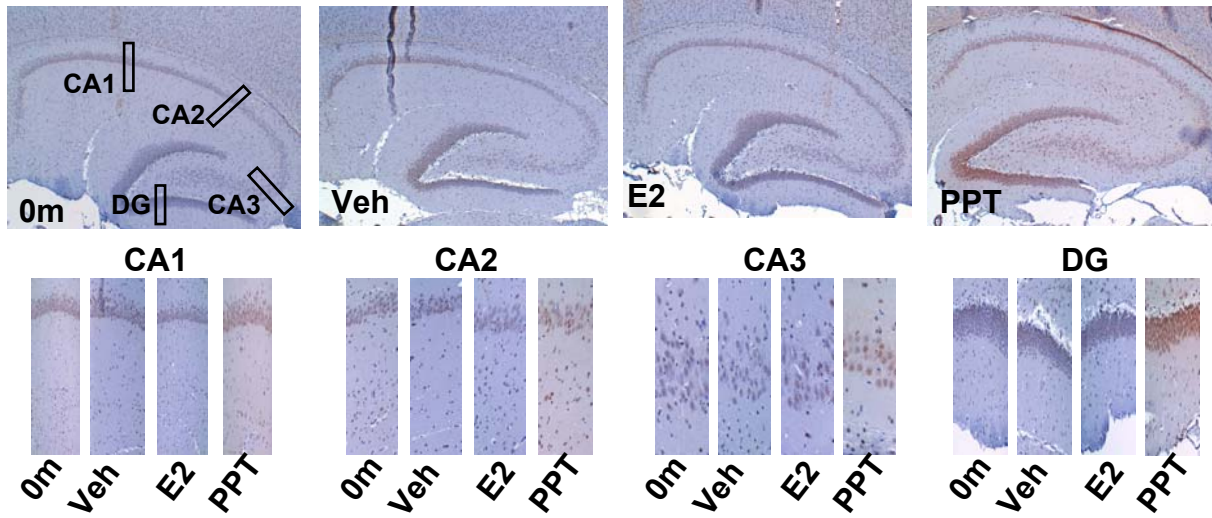


Figure 3. E2 induces the phosphorylation of ERK1/2, CREB, and p90RSK in a CaMKII- and Ca²⁺ influx-dependent manner. **a)** NLT cells were either unstimulated or treated with E2 (10nM), vehicle (0.1% EtOH), E2+ICI (10nM, 1uM), E2+BAPTA-AM (10nM, 10uM), E2+W7 (10nM, 10uM), E2+Nifedipine (10nM, 10uM), E2+U0126 (10nM, 10uM), PPT (100nM), PPT+ICI (100nM, 1uM), PPT+Nifedipine (100nM, 10uM), or PPT+U0126 (100nM, 10uM) for 10 min. All of the inhibitors were applied to the cells 30 minutes prior to E2 treatment. Immunoblotting was then used to detect phosphorylated levels of ERK1/2, CREB, and p90RSK, as well as pan Actin. **b)** NLT cells were treated with vehicle (0.1%EtOH), E2 (10nM), or E2+U0126 (10nM, 10uM, 30m pretreatment) for 10 min. Immunoblotting was used to detect autophosphorylated αCaMKII and phosphorylated levels of ERK1/2, ELK1, and CREB, as well as pan Actin.

Figure 4

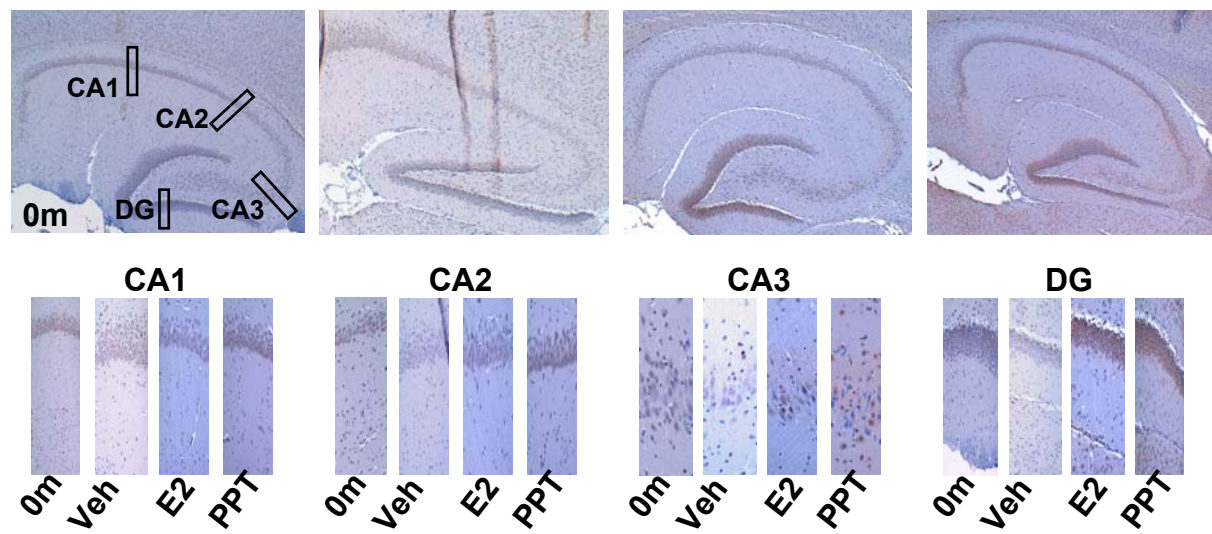
A.

15m



B.

1hr



C.

24hr

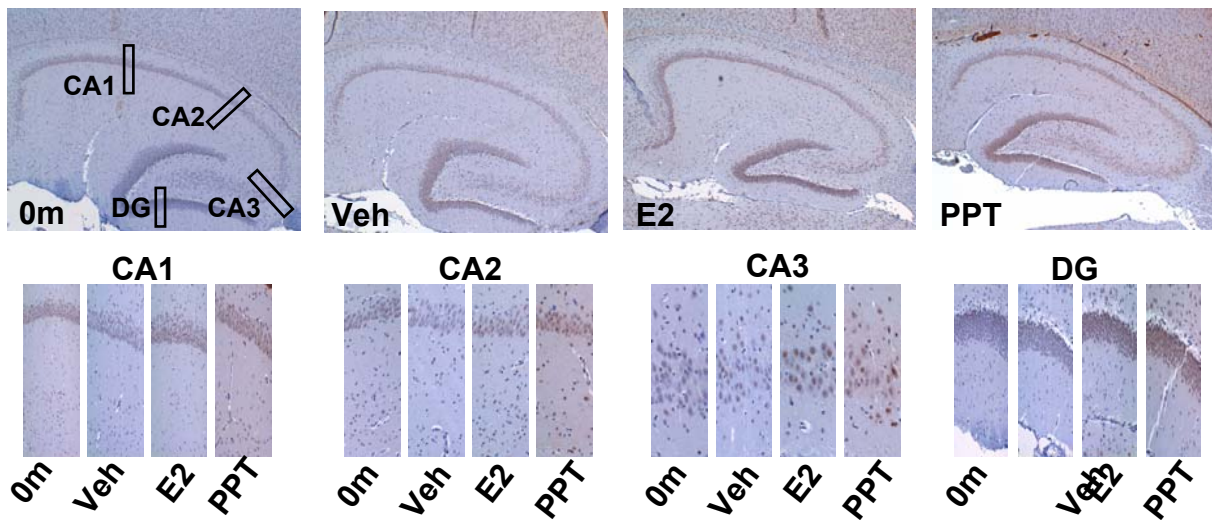


Figure 4. E2 induces α CaMKII autophosphorylation in rat hippocampus. **a-c)** Ovariectomized female rats were administered vehicle (10% cremaphor/2% EtOH in saline), E2 (0.2ug/rat), or PPT (10ug/rat) subcutaneously for **a)**15m, **b)**1hr, or **c)**24hr and then the brain tissue was harvested and immunohistochemistry was performed to detect autophosphorylated α CaMKII. Top panels represent 4x images of hippocampus. Bottom panels represent 20x images of CA1, CA2, or CA3 pyramidal neurons or dentate gyrus (DG). **d)** Ovariectomized female rats were injected intraperitoneally with saline or E2 (0.2ug/rat) and the brain was extracted at the indicated time points. Immunoblotting was used on the homogenized tissue to detect autophosphorylated α CaMKII and total α CaMKII levels. Graphic representation of all 6 replicates shown on right.

Figure 5

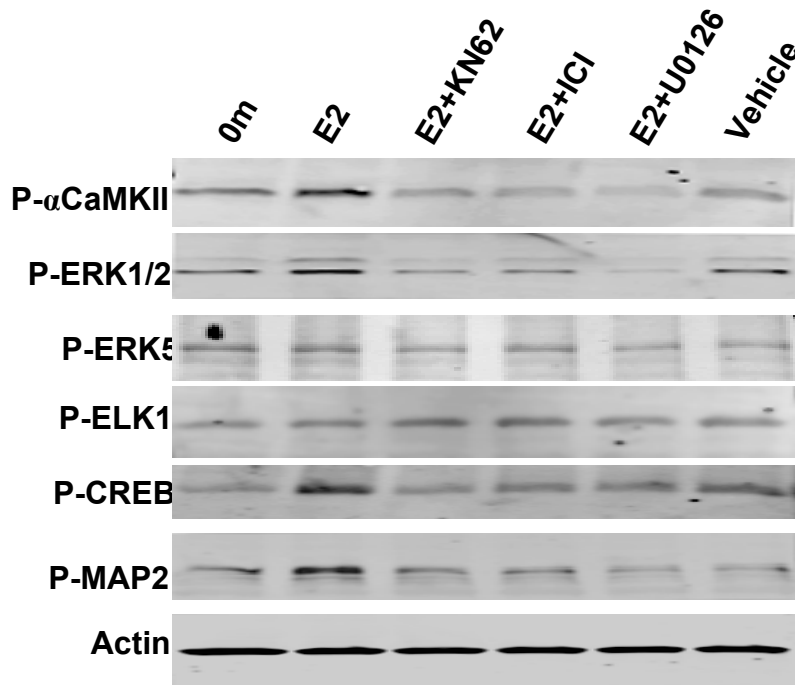


Figure 5. E2 induces phosphorylation of ERK1/2, CREB, and MAP2 in primary hippocampal neurons. Primary hippocampal cells were either unstimulated or treated for 10 min with vehicle (0.1%EtOH), E2 alone (1nM), or E2 (1nM) with 30 min pretreatment of KN62 (20uM), ICI (2uM), or U0126 (10uM). Immunoblotting detected phosphorylated levels of αCaMKII, ERK1/2, ERK5, ELK1, CREB, and MAP2 as well as pan Actin.