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UNIVERSITY OF PISA

DEPARTMENT OF REPRODUCTIVE MEDICINE AND CHILD DEVELOPMENT

PH.D. THESIS

IN

"PHYSIOPATHOLOGY OF REPRODUCTION AND SEXOLOGY"

UNIFIED MECHANISM OF ESTROGEN ACTION ON CENTRAL NERVOUS SYSTEM

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To those who have encouraged, supported and realized my dreams

CONTENTS

AKNOWLEDGEMENT

The data of this PhD thesis have been collected in almost two years experience in the laboratory of Roberta Diaz Brinton, University of California, Los Angeles and this paper will be published in an important international journal pretty soon. I want to thank Robbie because she has been an incredible mentor, a wonderful teacher and a good friend. She has an important and priceless role in this work and I will be always grateful. In her laboratory, I met good colleagues and above all, great friends. I'll never forget Jerome, Anna, Rana, Sean, Ron, Jon, Liqin, JunMing, Tzu-wei, Shuhua, Tino, Rick and Jeanette. Your names, your smiling faces and your supporting words will be impressed on my heart, forever.

In this section, I cannot forget the basis of my education, Dr Tommaso Simoncini and Prof. Andrea Riccardo Genazzani. They are leading me to the highest levels of knowledge for a medical student and doctor. They have been always next to me, supporting my dreams, solving my doubts and making real something which was only in my heart.

Finally, I want to remember my family and in particular Federica. You have always encouraged me, my dreams, my life. I never felt to be alone not even thousands miles away and all your love has been so far…so close. Thanks.

SUMMARY

17β-estradiol (E_2)-induced neuroprotection is dependent upon both mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling cascades. We sought to determine whether E_2 neuroprotective mechanisms are through a unified signaling cascade activated by estrogen receptor (ER)-PI3K interaction within the same population of neurons or whether E_2 activation of ERK1/2 and Akt are independent signaling events in different neuron populations. Immunoprecipitation of E_2 treated cortical neurons was conducted to determine a protein-protein interaction between ER and p85, the PI3K regulatory subunit. Subsequently, cortical neurons were treated with E_2 alone or in presence of MAPK inhibitors or PI3K inhibitors. Results of these analyses indicated a protein-protein interaction between ER and p85 that was time-dependent and consistent with the temporal profile for generation of both Akt (pAkt) and ERK 1/2 phosphorylation (pERK 1/2). E_2-induced phosphorylation of Akt, was first apparent at 10' and maximal at 30'. Simultaneously, E_2 induced pERK 1/2 was first apparent at 5-10' and maximal at 30'. Inhibition of PI3K completely blocked E_2 activation of pAkt at 10' and 30'and blocked E_2 activation of ERK1/2 at 10'. Double immunocytochemical labeling for pERK 1/2 and pAkt showed that E_2 induced both signaling pathways in the same neurons. These results indicate a unified signaling mechanism of E_2 action that is initiated by an interaction between ER and p85 and which leads to the coordinated activation of both pERK 1/2 and pAkt in the same population of neurons. These results are considered from an estrogen mechanism of action and a therapeutic development perspective.

INTRODUCTION

Estrogen effects in the central nervous system are multifaceted and encompass mechanisms that range from chemical, biochemical and genomic (Brinton, 2001). Activation of these mechanisms is associated with estrogen promotion of neuronal plasticity (Woolley and McEwen, 1992; Brinton, 1993; Hao et al., 2003), increased cell survival (Brinton et al., 1997) neuronal development (Toran-Allerand, 1984, 1991; Miranda et al., 1994), regulation of apoptosis (Dubal et al., 1999; Pike, 1999; Kaja et al., 2003; Nilsen and Brinton, 2003b) and protection against toxic insults (Gustafsson, 1997; Simpkins et al., 1997; Diaz Brinton et al., 2000). The neuroprotective outcomes of E_2 fall within three broad functional categories; antioxidant, defense, and viability (Brinton, 2001). The extent of these functions has not been fully determined, however each appear to make a significant if not obligatory impact on the neuroprotective efficacy of estrogens.

Mechanistically, E_2 activates a myriad of signaling cascades in neurons, including mitogen-activated protein kinase (MAPK) (Watters et al., 1997; Singer et al., 1999; Singh et al., 1999; Nilsen and Brinton, 2003a), phosphatidylinositol-3-kinase (PI3K) (Singh, 2001; Zhang et al., 2001), G protein regulated signaling (Kelly et al., 2002), c-fos (Rudick and Woolley, 2000), protein kinase C (PKC) (Cordey et al., 2003) and Ca^{2+} influx (Cordey et al., 2003; Wu et al., 2005). Each of these have been associated with E_2 regulation of neuronal function and survival (Singh et al., 1994; McEwen et al., 1997; Mor et al., 1999; Woolley, 1999; Brinton, 2001; McEwen, 2001; Nilsen, 2002; Nilsen and Brinton, 2003b). Of these pathways, PI3K has the potential for activating a host of downstream signaling cascades (Yin et al., 1998; Simoncini et al., 2000; Alexaki et al., 2004; Ghisletti et al., 2005). In particular, PI3K recruitment plays a pivotal role in neuroprotection through downstream activation of Akt (Singh, 2001; Znamensky

et al., 2003), including E_2 mediated protection against β-amyloid or glutamate toxicity (Honda et al., 2000; Zhang et al., 2001). In parallel, E_2 dependent MAPK activation phosphorylates CREB, which increases transcription of genes related to morphogenesis, including spinophilin,(Zhao et al., 2004), and the anti-apoptotic proteins Bcl-2 (Nilsen and Brinton, 2003b) and Bcl-xl (Pike, 1999), required for neuroprotection. Both the pAkt and pERK 1/2 pathways are activated within minutes of E_2 exposure and have been associated with a membrane site of E_2 action (Nilsen and Brinton, 2003a; Toran-Allerand et al., 2005; Wu et al., 2005).

We sought to test the hypothesis that E_2 induces the coordinated activation of the pERK 1/2 and pAkt signaling cascades, as part of a unified signaling cascade in the same population of neurons. To test this hypothesis, we pursued the mechanism of E_2 activation of the PI3K signaling pathway by first determining if the neuronal estrogen receptors undergo a protein-protein interaction with the PI3K regulatory subunit p85. Second, we determined the requirement for PI3K for activation of both pAkt and pERK 1/2. We further determined the requirement of PI3K for E_2 activation and colocalization of pAkt and pERK 1/2.

METHODS

Chemicals. Neural culture materials were from Invitrogen. Steroids were dissolved in ethanol and diluted in culture medium with final ethanol concentration of <0.001%. PD 98059, UO126, UO124 and wortmannin were from Calbiochem. Concentration of E2 (10 nM), wortmannin (30 nM, IC50 5nM for PI3K (Arcaro and Wymann, 1993)), PD 98059 (20 μM, IC50 5 μM for MAPK (Borsch-Haubold et al., 1996)) UO126 (20 μM IC50 1 μM, IC50 (Liu et al., 2002)) and UO124 (20μM) were chosen based on literature research (Qiu et al., 1998; Zhu et al., 2004). Furthermore, to remove possible no specific inhibition of non target kinases, inhibitors concentration were adjusted based on Davies's study on kinase inhibitors where for exempla wortmannin shows to have an inhibition activity on MAPK <4% versus >99 % on PI3K (Davies et al., 2000).Cortical neurons were preincubated with inhibitors for 30' before starting each experiment.

Animals care. Use of animals was approved by the Institutional Animal Care and Use Committee in University of Southern California (protocol number 10256). Pregnant Sprague Dawley rats were purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN, USA. and housed under controlled conditions of temperature (22ºC), humidity, and light (14 hour light: 10 hour dark); water and food were available ad libitum.

Neuronal Culture. Primary and pure cultures of cortical neurons were obtained from Embryonic Day 18 (E18d) rat fetuses as previously described (Nilsen and Brinton, 2003a). Briefly, cortical cells were dissected and treated with 0.02% trypsin in HBSS (Invitrogen, Grand Island, NY) for 5 min at 37°C, and dissociated by repeated passage through a series of fire-polished constricted Pasteur pipettes. Cells were plated on poly-D-lysine-coated 60mm Falcon Petri dishes at a density of 0.5–1 x 10^5 cells/cm^2 for biochemical study. Cells were plated on NalgeNunc

(Naperville, IL) CC_2-coated four-well chamber slides at a density of 2–4 x 10^4 cells/cm^2 for morphological study. Neurons were grown in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 10 U/ml penicillin, 10 µg/ml streptomycin, 0.5 mM glutamine, 25 µM glutamate, and 2% B27 (Invitrogen, Gaithersburg, MD). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. All experiments were performed at the times indicated.

Western Blotting. Cells were washed with cold PBS and incubated in ice-cold lysis buffer consisting of 0.1% SDS, 1% Igepal CA-630 (nonionic, non-denaturing detergent), 0.2mM phenylmethylsulfonylfluoride, and 0.01‰ protease inhibitor mixture (Sigma) for 30 min at 4°C. Cell lysates were centrifuged at 12,000g for 10 min, and the concentration of protein in the supernatant was determined by the BCA protein assay (Sigma). Twenty micrograms of total protein from whole-cell lysates were separated under reducing and denaturing conditions by 10% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore). Nonspecific binding sites were blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-Tween) for 30' at RT. Membranes were then incubated with anti phospho ERK 1/2 antibody (1:750 in PBS-T/1% horse serum, Cell Signaling Technologies, Beverly, MA; PBS-T is PBS containing Tween) or total ERK 1/2 antibody (C-14) (1:5,000 in PBS-T/1% horse serum; Santa Cruz Biotechnology) or anti-phospho AKT (1:1000 in PBS-T/1% horse serum; Cell Signaling Technologies) or anti-total Akt (1:500 in PBS-T/1% horse serum; Cell Signaling Technologies) overnight at 4°C. Membranes were then incubated in horseradish peroxidase-conjugated goat anti-rabbit (1:5000) or horse anti-mouse IgG (1:3000) for 1 hour at RT, and results were visualized by the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Relative amounts of pERK 1/2, pAkt, total ERK 1/2 and total pAkt were quantified by optical

density analysis using the UN-SCAN-IT gel automated digitizing system (Scion, Frederick, MD). Data are presented as means ± SEM for at least three independent experiments.

Immunoprecipitations. Cells were washed with ice-cold PBS and lysed with the following buffer: Tris-HCl (20 mM, pH 7.4), EDTA (10 mM), NaCl (100 mM), IGEPAL (1%), Na3VO4 (1 mM), NaF (50 mM), PMSF (0.1 mg/l), aprotinin (0.3 mg/l) and 0.01‰ protease inhibitor cocktail (Sigma). The immunoprecipitating antibody (1 mg) was added to equal amounts of cell lysates (0.5±1 mg) in 500ml of lysis buffer for 1 h at 4 °C with gentle rocking. Afterwards, 40 ml of 1:1 Protein-A-agarose were added to the entire mixture and rocked gently for an additional hour at 4 °C. The mixture was then centrifuged at 12,000g for 5 min at 4 °C. The supernatant was removed and the immunoprecipitate was washed three times with 500 ml of washing buffer, which is composed of Tris-HCl (20 mM, pH 7.4), EDTA (10 mM), NaCl (150mM), IGEPAL (1%), Na3VO4 (1 mM), NaF (50 mM), PMSF (0.1 mg/l), aprotinin (0.3 mg/l) and 0.01‰ protease inhibitor cocktail (Sigma). Immunoprecipitated proteins were separated under reducing and denaturing conditions by 10% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore). Nonspecific binding sites were blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-Tween). Membranes were incubated with anti-ER (1:250, Ab-10: Clone TE111.5D11, NeoMarkers, Fremont, CA) or anti-p85a (Upstate Biotech., Lake Placid, NY) antibody. After four 10 minute washes in PBS, membranes were incubated in horseradish peroxidase-conjugated anti-mouse IgG (1:3000), and results were visualized by the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Relative amounts of ER and p85 were quantified by optical density analysis using the UN-SCAN-IT gel automated digitizing system (Scion, Frederick, MD).

Immunocytochemistry. Cortical neurons cultured on gridded coverslips (Fisher scientific, Pittsburgh, PA, USA) were exposed to E_2 (10 nM) at different time points and paraformaldehyde (4%)-fixed. Fixed neurons were permeabilized with 0.5%Triton/PBS. Cells were incubated with anti phospho ERK 1/2 antibody (1:300; Cell Signaling Technologies) and anti-phospho AKT (1:250, Cell Signaling Technologies) for 3 hours at RT followed by incubation, respectively, in fluorescein-conjugated goat anti-rabbit secondary antibody (1:250, Vector Laboratories) and in Cy3 (1:1000, Amersham) for 1 hour at RT. Cells were mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Images were taken and relative immunoreactive intensity was calculated with Slidebook Digital Imaging System (Intelligent Imaging Innovations, Inc, Denver, CO, USA). The area of DAPI staining was mapped onto the FITC images to define the nucleus as the region of interest or as a mask to define the cytoplasm as the region of interest. The relative fluorescence intensities of 20 randomly selected cells were normalized to the average fluorescence intensity of control cells and presented as mean ± SEM. The cytoplasm and nucleus were analyzed independently of each other.

Unbiased analysis of pERK 1/2 and pAkt positive cortical neurons. pERK 1/2 and pAkt positive neurons were detected and quantified using 3i Slidebook 4.1 software (Intelligent Imaging Innovations, Denver, CO). For each condition, microscopic fields were randomly selected and over 2000 cells across 3 separate experiments were analyzed for pAkt and/or pERK 1/2 immunoreactivity using an Axiovert 200 M Marianas Digital Microscopy Workstation (Intelligent Imaging Innovations, Denver, CO). A binary overlay on a 2D mask was used for each fluorescent marker (DAPI, FITC and Cy3) to perform an advanced

selection of a set of random regions at the same resolution for image analysis. Subsequently, morphometric measurements (area, volume and estimated surface and perimeter) and fluorescent intensity (mean, minimum and maximum, colocalization) were performed.

Statistics. Statistically significant differences between groups were determined for all assays by an one way ANOVA followed by a Newman–Keuls post hoc analysis. Data derived from Western blot analyses represent semiquantitative estimates of the amount of a specific protein that was present in a cell extract. The data displayed in graphs are reported as means ± SEM or fold change ± SEM of the actual scanning units derived from the densitometric units normalized to internal standard for protein content.

RESULTS

Protein/protein interaction between Estrogen Receptor (ER) and the PI3K regulatory subunit p85

To investigate the mechanism by which E_2 activates PI3K, immunoprecipitation of ER was performed followed by Western blot for p85, the regulatory subunit of PI3K (Fig 1A). To optimize the probability of detecting a protein/protein interaction with ER, we used an ERα selective antibody (clone TE111.5D11), which is known to also recognize ERβ. The TE111.5D11 clone has greater affinity for ERα, but because it was developed against the entire ER LBD which has 50-60% homology between ERα and ERβ, it partially recognizes also ERβ (Abbondanza et al., 1993; Mosselman et al., 1996). We reasoned that the use of a LBD region specific antibody had the probability of enriching the pool of potential ERs binding to p85.

Following E_2 treatment, ER immunoreactivity was first apparent in the p85 immunoprecipitate at 10', returned to baseline at 20' and was restablished at 30' when the protein/protein interaction was maximal (see Fig. 1A). These results were confirmed by immunoprecipitation of p85 followed by Western blot analysis for ER (see Fig. 1B). As shown in Fig. 1A, immunoreactivity for p85 in the immunoprecipitate of ER, was initially apparent at 10', maximal at 30' and returned to baseline by 45'. The temporal pattern of protein/protein interaction between ER and p85 suggests an initial coupling at 10' followed by decoupling between 11-20' followed by a recoupling at 30' and an uncoupling between 31-45'. These findings suggest a dynamic shuttling of ER and p85 from uncoupled to coupled states in the presence of E_2 in cortical neurons.

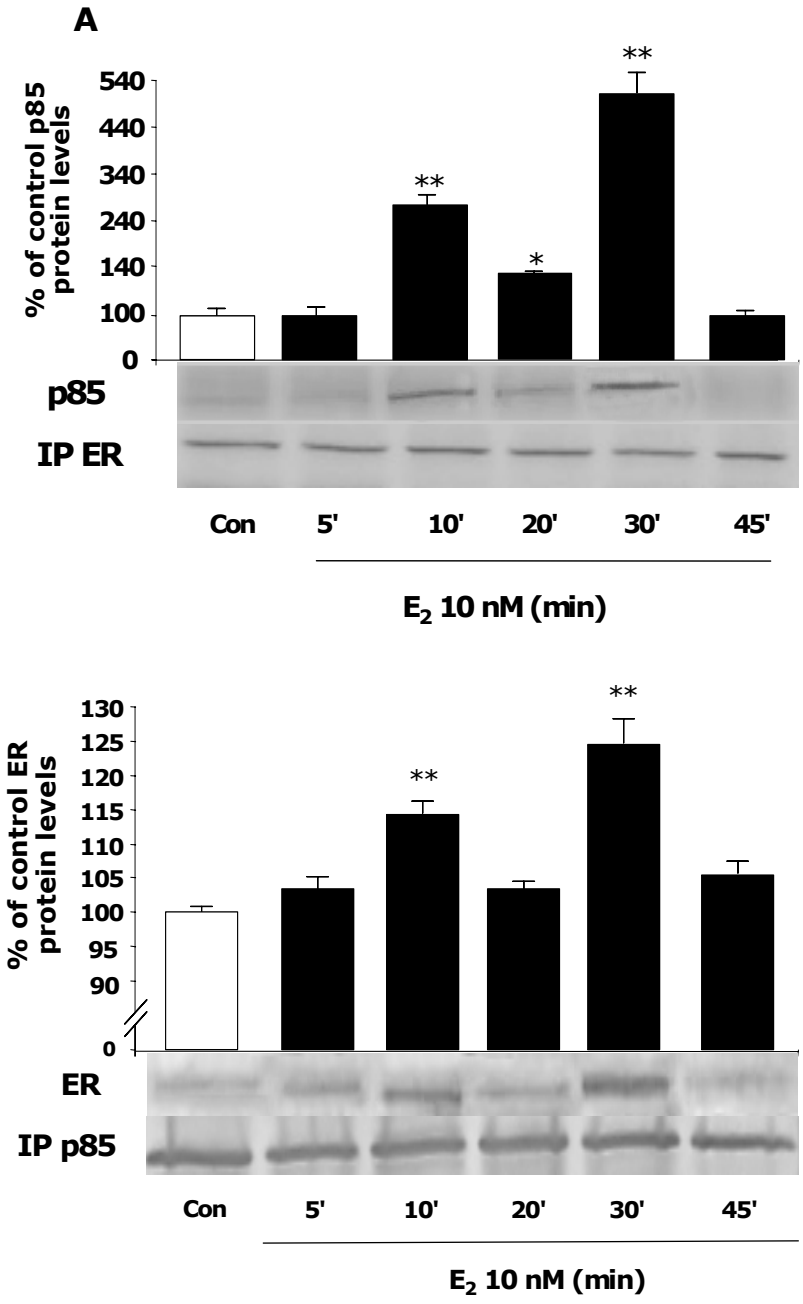


Figure 1 A-B. ER physically interacts with p85, PI3K subunit in a time dependent manner.

Rat cortical neuron cells were exposed to E_2 (10 nM) at different time points (0', 5', 10', 20', 30' and 60'). Immunoprecipitation of Estrogen Receptor (ER) was performed and followed by

Western blot to p85, the regulatory subunit of PI3K (Fig 1A). p85 has a maximal interaction with ER at 10' and 30'. To confirm these data we repeated the same experiment immunoprecipitating p85 and Western blot to ER (Fig 1B). *, $P < 0.05$ vs. control neurons; **, $P<0.01$ vs. control neurons. Data shown are from a single experiment and are representative of 3 independent experiments.

E_2 increased Akt and ERK 1/2 phosphorylation in a time-dependent manner

To determine the downstream consequence of ER/p85 protein/protein interaction, rat cortical neurons were exposed to E_2 (10 nM) at different time points (0',5', 10', 20', 30', 45' and 1 hour) and protein was harvested for Western blot analyses for pAkt and pERK 1/2. Activation of pAkt and pERK 1/2 were detected with phosphorylated residue selective antibodies (Fig. 2A-B). Consistent with the protein/protein interaction time course between ER and p85, E_2 maximally induced Akt at 10' and 30' and was uncoupled at 20' (Fig. 2A). In parallel to the pAkt activation, E_2 significantly induced ERK 1/2 phosphorylation at 5' which peaked at 30' (Fig. 2B). As a control, the same membranes were reblotted for total Akt and ERK 1/2 and no difference in protein loading was observed (Fig. 2A-B).

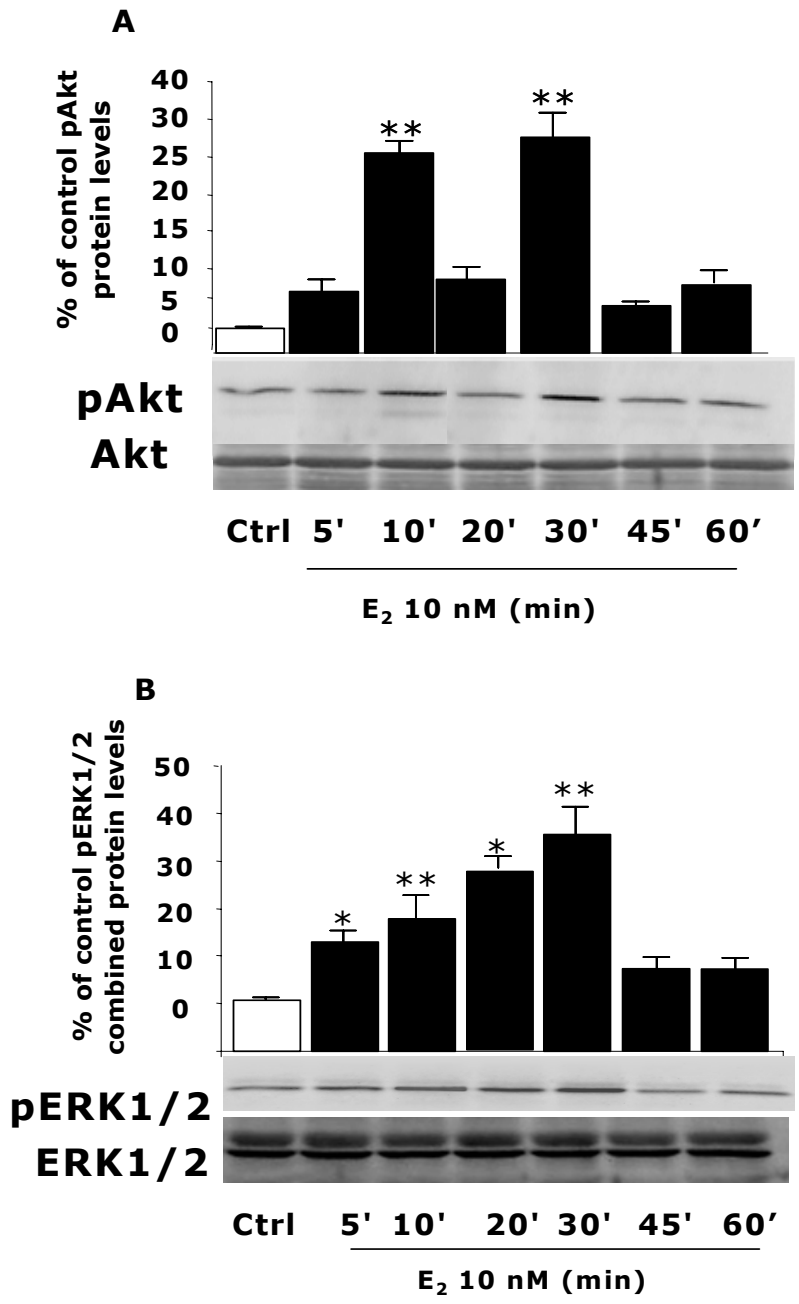


Figure 2 A-B. E_2 induces Akt and ERK 1/2 phosphorylation in a time dependent manner.

Rat cortical neuron cells were exposed to E_2 (10 nM) at different time points (0',5', 10', 20', 30', 45' and 60'). Western Blot analysis shows E_2 induces AKT phosphorylation with a maximum efficacy at 10' and 30' (Fig. 2A). ERK 1/2 phosphorylation is induced at 5'-10' with a maximal

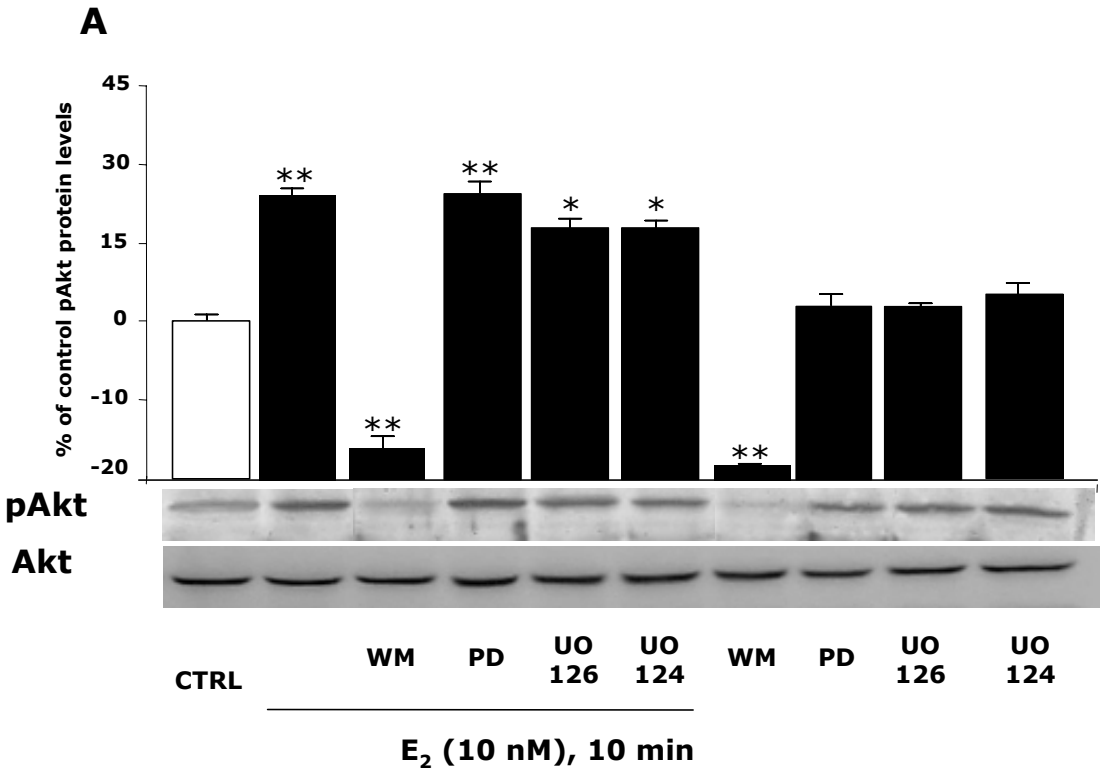
activation at 30' (Fig. 2B). As a loading control we reblotted the same membrane with the total form of AKT and ERK 1/2 and the densitometry of phosphorylated forms were normalized in base of those. No differences in expression were found. *, $P < 0.05$ vs. control neurons; **, $P<0.01$ vs. control neurons. Data shown are from a single experiment and are representative of 3 independent experiments.

At 10 min inhibition of PI3K blocked E_2 activation of both pAkt and pERK 1/2

To determine whether PI3K was required for E_2 activation of both pAkt and pERK 1/2, cortical neurons were treated with a PI3K inhibitor, wortmannin (30 nM), two different MAPK pathway inhibitors, PD 98059 (20 μM) and UO126 (20 μM), or the UO126 negative control, UO124 (20 μM), alone or with E_2 (10 nM) for 10'. To eliminate crosstalk between the PI3K and MAPK signaling pathways, concentrations of inhibitors that generated maximal inhibition were used (Qiu et al., 1998; Zhu et al., 2004).

As expected, E_2-dependent Akt phosphorylation was blocked by the PI3K inhibitor, wortmannin (Fig 3A) whereas MAPK inhibitors did not block E_2-induced pAkt. However blockade f PI3K pathway by wortmannin, completely blocked E_2-induced ERK 1/2 activation (Fig 3B). As anticipated, E_2-induced pERK1/2 was blocked by the MAPK pathway inhibitors, PD 98059 and UO126. The negative control for MEK inhibition, UO124, was without effect alone and did not block E_2-induced pAkt and pERK 1/2. Inhibition of PI3K by wortmannin blocked E_2-induced phosphorylation of both pAkt and pERK (Fig 3A-3B), however wortmannin alone completely blocked phosphorylation of Akt while preventing E_2-induction of ERK 1/2 activation above baseline. These data indicate that wortmannin is a selective inhibitor of only

PI3K and does not affect the MAPK cascade directly. In this instance, wortmannin inhibited the MAPK pathway only as it was activated by E_2-induced PI3K activation. It is important to note that the MAPK inhibitors PD and UO126, alone and with E_2, did not significantly interfere with PI3K activation of Akt as MAPK is downstream to PI3K.



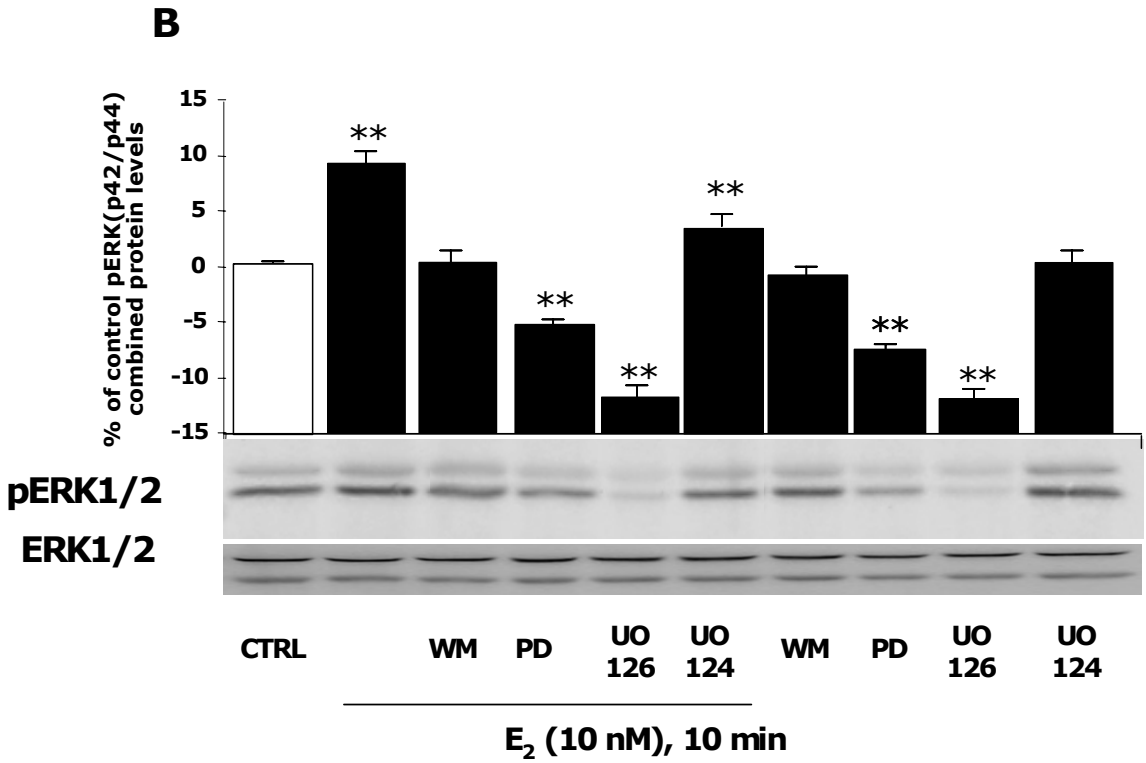


Figure 3 A-B. E_2 induces rapid Akt and ERK 1/2 phosphorylation but the blockade of PI3K blocks pERK 1/2 estrogen induced at 10'. Rat cortical neurons were treated with a phosphonositide PI3K inhibitor (wortmannin, WM), two different MAPK inhibitors (PD 98059 and UO126), or a positive control of MAPK (UO124) alone or with E_2 (10 nM) for 10'. Western blot analysis shows inhibition of PI3K with wortmannin completely blocks E_2 activation of both pAkt (Fig. 3A) and pERK 1/2 (Fig. 3B) at 10'. As a loading control we reblotted the same membranes with the total form of AKT and ERK 1/2 and the densitometry of phosphorylated forms were normalized in base of those. No differences in expression were found. *, $P < 0.05$ vs. control neurons; **, $P<0.01$ vs. control neurons. Data shown are from a single experiment and are representative of 3 independent experiments.

At 30 min inhibition of PI3K completely blocked E_2 activation of pAkt while partially inhibiting E2 dependent pERK 1/2

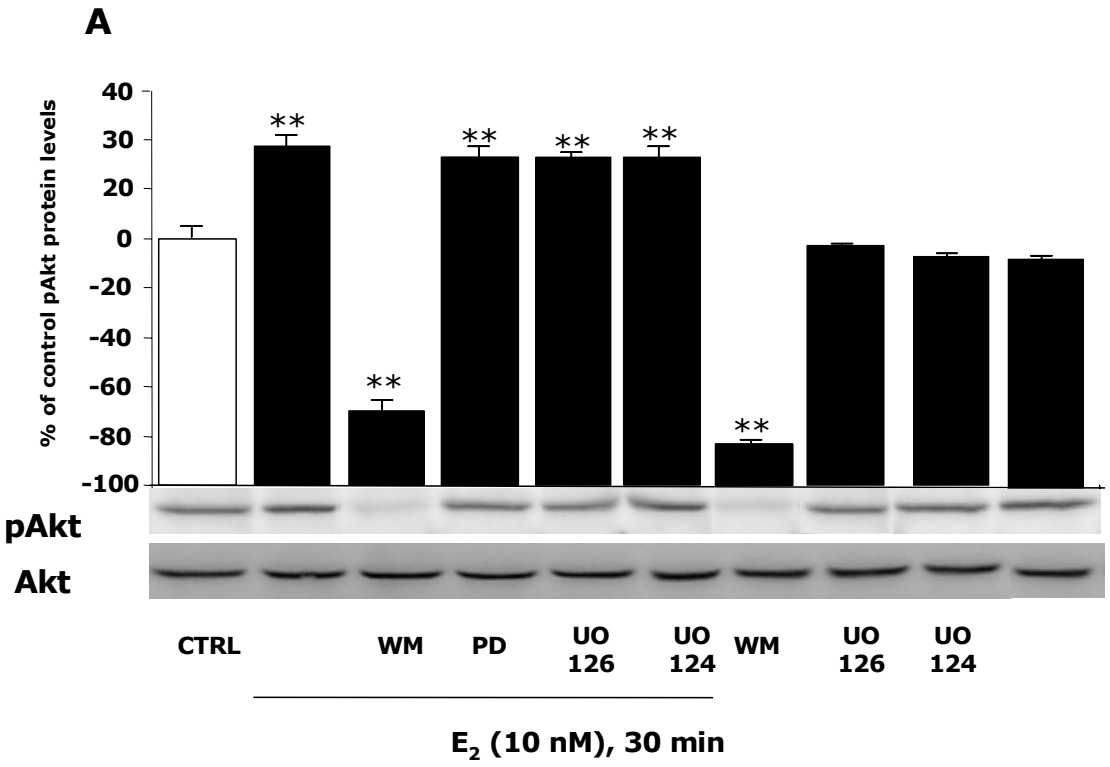
To determine the relationship between E_2 activation of PI3K and subsequent activation of the early (10') versus late (30') phases of pAkt and pERK 1/2, cortical neurons were treated with a PI3K inhibitor (wortmannin), two different MAPK pathway inhibitors (PD 98059 and UO126), or a negative control for UO126 (UO124) alone or with E_2 (10 nM) for 30'. As excepted, E_2-dependent Akt phosphorylation was completely blocked by wortmannin at 30', while MAPK inhibitors did not significantly inhibit Akt phosphorylation (Fig 4A).

As previously shown, E_2 induced ERK 1/2 phosphorylation at 30'. This phosphorylation was completely blocked at 30' by MAPK inhibitors (PD 98059 and UO126), whereas PI3K inhibitor (wortmannin) partially blocked ERK 1/2 activation (Fig. 4B), in contrast to wortmannin completely inhibiting pERK 1/2 at 10'.

To estimate potential induction of ERK 1/2 signaling pathway by PI3K during the 30' exposure, activation of each pathway was completely abolished with inhibitors at the same concentration indicated earlier. It is important to note that wortmannin did not block E_2-dependent ERK 1/2 phosphorylation at 30' and that the reduction of pERK 1/2 by E_2 plus wortmannin is only 5-7%. Moreover, wortmannin alone did not alter the baseline level of MAPK at 30', consistent with previous data at 10'.

To confirm that inhibition of PI3K at 30' was achieved, we used a second PI3K inhibitor LY294004, and determined the consequences of inhibiting PI3K on E2-inducible Akt and ERK 1/2 activation at10' and 30'. Western blot analysis indicated that like wortmannin, LY294004 completely blocked E2 activation of both pAkt (Fig. 4C) and pERK 1/2 (Fig. 4D) at 10'. At 30',

wortmannin and LY294004 completely blocked E2 activation of Akt while not inhibiting E2 activation of pERK1/2. These data confirm previous results in which inhibition of PI3K blocked E2-inducible ERK1/2 at 10' and not at 30'. Collective, these data indicate that PI3K regulates the initial component of MAPK activation whereas inhibition of PI3K over the course of 30' results in an E_2-dependent and PI3K-independent activation of neurons.



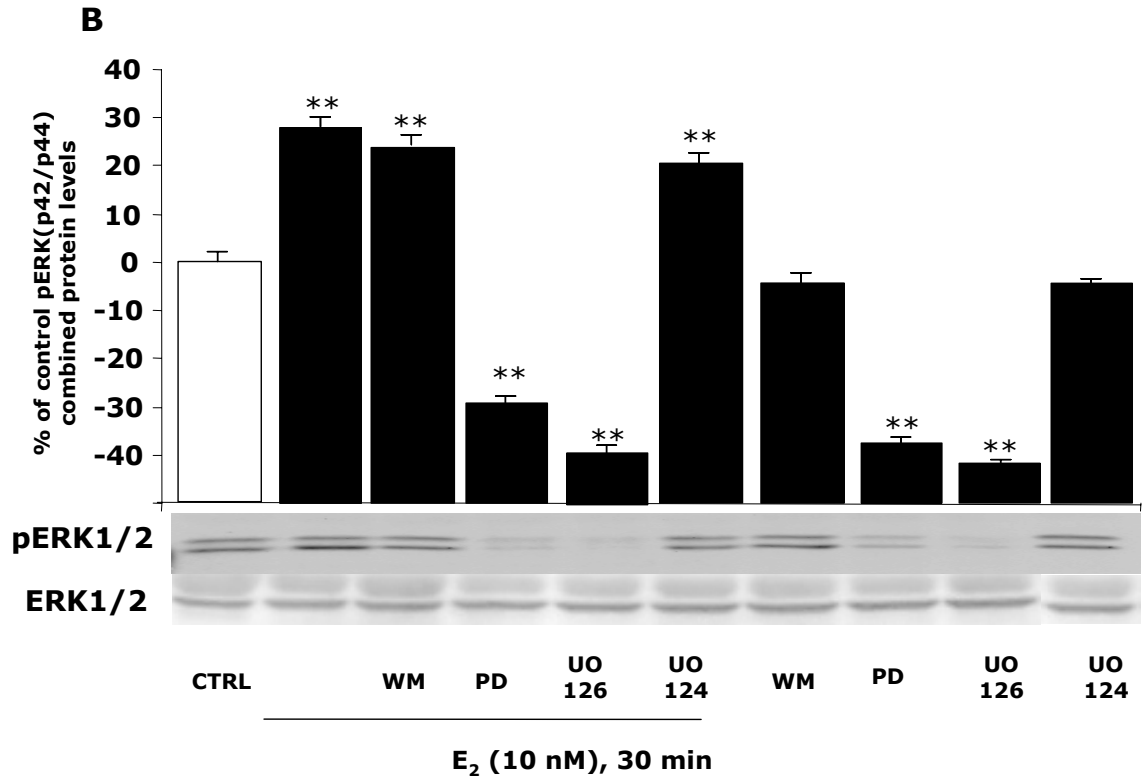
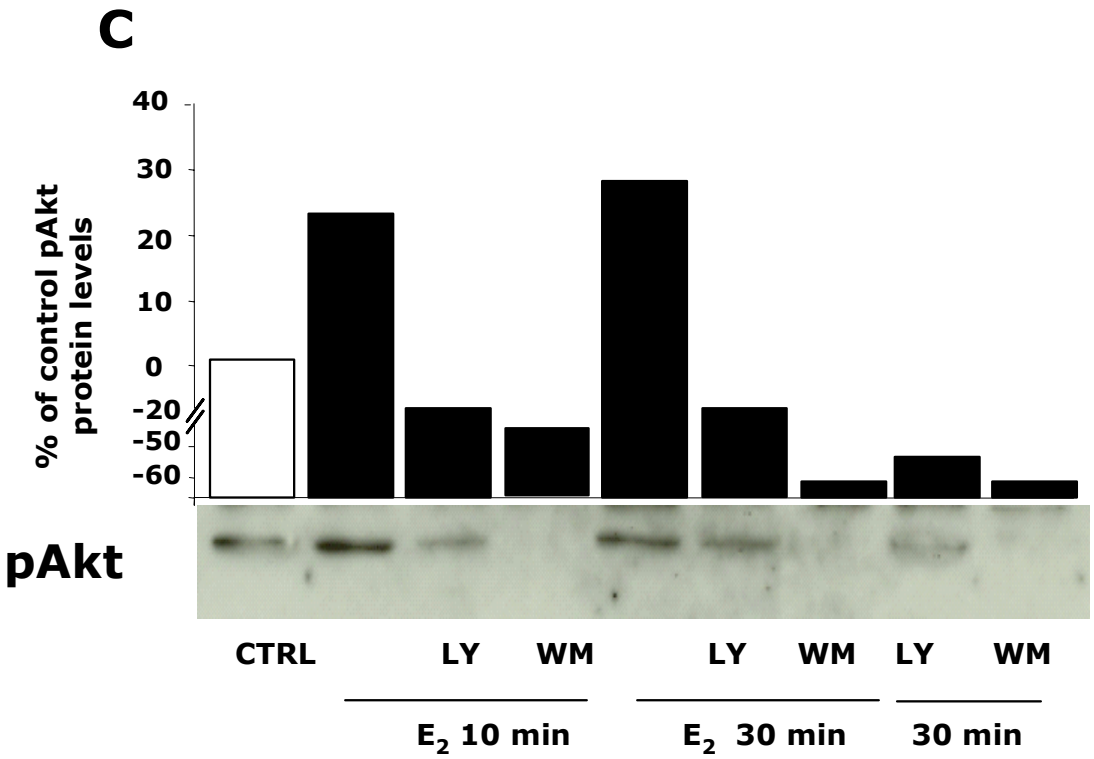


Figure 4 A-B. Inhibition of PI3K completely blocks E_2 activation of pAkt but not E_2 activation of pERK 1/2 at 30'. Cortical neurons were treated with a PI3K inhibitor (wortmannin, WM), two different MAPK inhibitors (PD 98059 and UO126), and a positive control of MAPK (UO124) alone or with E_2 (10 nM) for 30'. E_2-dependent Akt phosphorylation was blocked by wortmannin at 30' but the use of MAPK inhibitors only partially interfered with Akt phosphorylation (Fig. 4A). ERK 1/2 phosphorylation is completely blocked by MAPK inhibitors (PD 98059 and UO126) while the use of wortmannin did not prevent ERK 1/2 activation as it had at 10' (Fig 4B). As a loading control we reblotted the same membrane with the total form of AKT and ERK 1/2 and the densitometry of phosphorylated forms were

normalized in base of those. No differences in expression were found. *, P < 0.05 vs. control neurons; **, P<0.01 vs. control neurons. Data shown are from a single experiment and are representative of 3 independent experiments.



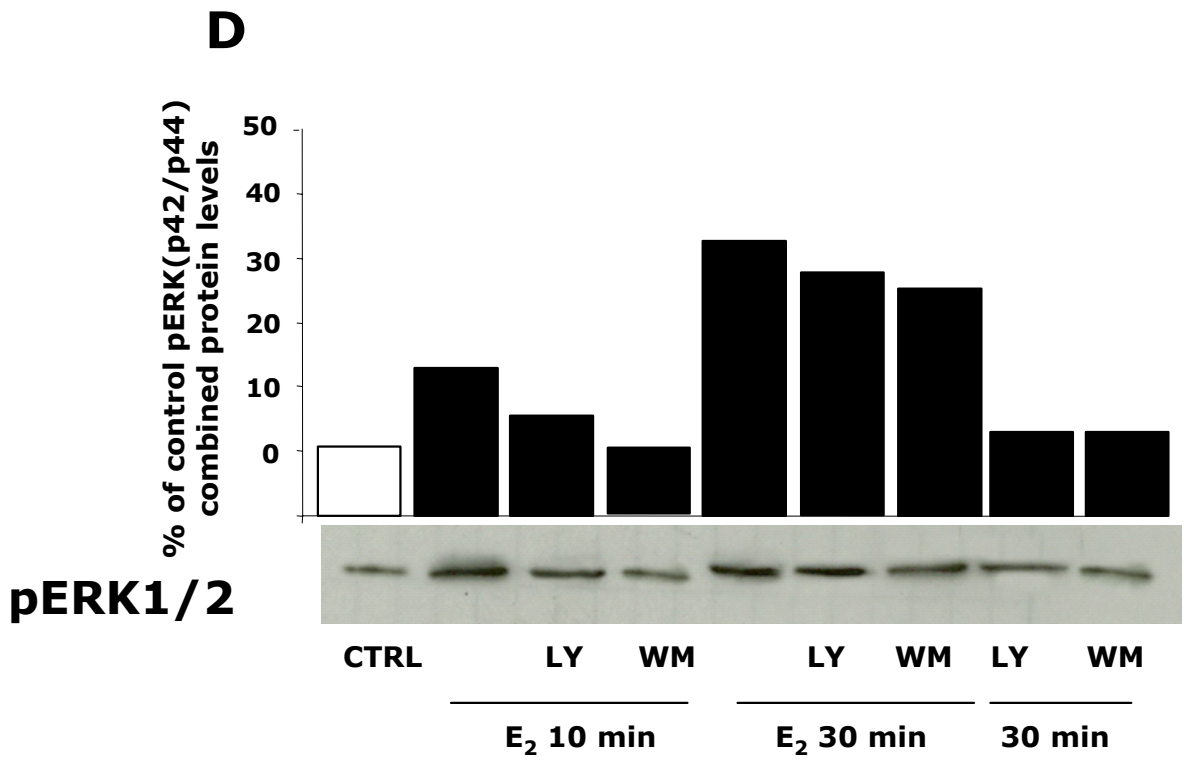


Figure 4 C-D. Wortmannin is more potent PI3K inhibitor than LY294004. To compare the effect of wortmannin and LY294004 on Akt and ERK 1/2, cortical neurons were treated with PI3K inhibitors (wortmannin and LY294004) alone or with E_2 (10 nM) for 10' and 30'. Western blot analysis shows that wortmannin and LY294004 completely block E_2 activation of both pAkt (Fig. 4C) and pERK 1/2 (Fig. 4D) at 10'. At 30', wortmannin and LY294004 completely block E_2 activation of Akt and only partially interfere with E_2 dependent ERK 1/2 activation. Both blots suggest that wortmannin is a more potent PI3K inhibitor than LY294004 of Akt and ERK 1/2 activation at 10' and 30'. Data shown are from a single experiment and are representative of 2 independent experiments.

E_2-dependent Akt and ERK 1/2 phosphorylation occurred in the same population of cortical neurons in a time-dependent manner

To determine whether E_2 induced pAkt and pERK 1/2 occurred in the same or in independent populations of neurons, cortical neurons were exposed to E_2 (10 nM) at different time points (0', 10', 20'and 30') and double labeled immunocytochemically for pAkt and pERK 1/2 at each time point (Fig 5A-B). For each condition, microscopic fields were randomly selected and over 2000 neurons across 3 separate experiments were analyzed for pAkt and/or pERK 1/2 immunoreactivity (Fig 5A). Quantitative computational threshold analysis of pAkt and pERK 1/2 positive cells was conducted blind to experimental condition (Fig 5A). Following 10' of E_2 exposure, 42% of neurons were positive for pAkt, 27% were positive for pERK 1/2 and 25% were positive for both pAkt and pERK. At 20', the number of neurons positive for pAkt, pERK 1/2 and co-expressing pAkt and pERK were essentially equal at 35%. At 30', when both pAkt and pERK 1/2 activation were maximal, 68% of neurons were positive for pAkt and 63% of neurons were positive for pERK 1/2. Also at 30', pERK 1/2 and pAkt were co-expressed in nearly the majority of the population with over 62% of neurons simultaneously activated (Fig 5A). These data are consistent with the Western blot data which shows that E_2 induced an increase in Akt and ERK 1/2 phosphorylation at 10' and 30'.

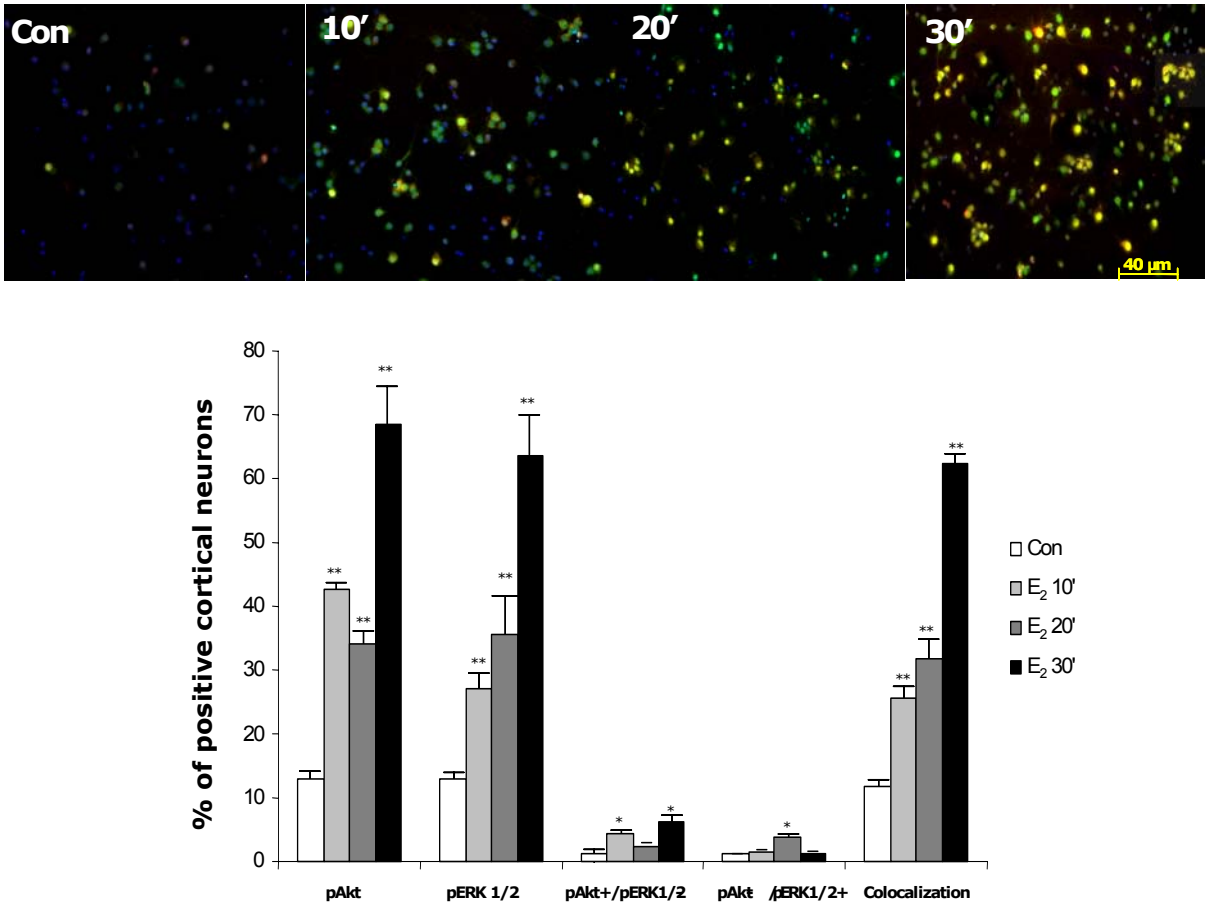


Figure 5A. Estrogen-dependent Akt and ERK 1/2 phosphorylation within the same cortical cell in a time-dependent manner. Rat cortical neuron cells were exposed to E_2 (10 nM) at different time points (0', 10', 20' and 30'). pERK 1/2 (red) pAkt (green) pAkt/pERK 1/2 colocalization (yellow) immunofluorescence and DAPI nuclear counterstain (blue) E_2 induced Akt and ERK 1/2 phosphorylation whose fluorescent intensity is maximal at 10' and 30' in the same cells as we excepted from WB analysis. Fluorescent microscopic images show morphology and relative density of pAkt and pERK 1/2 positive cells in the E_2 treated group versus control (time 0'). Data shown are from a single experiment and are representative of 3 independent

experiments. After immunochemistry, counting of E_2 dependent activated cells for Akt or ERK 1/2 or both was performed for each microscope field in comparison with non-treated cells. E_2 treatment induced an increasing and time-dependent neuron activation with a maximum at 30'. Bar graph depicts the percentage of activated cells for Akt or ERK 1/2 or both in the E_2 group versus control (time 0'). *, $P < 0.05$ vs. control neurons; **, $P<0.01$ vs. control neurons. Data shown are from a single experiment and are representative of 3 independent experiments.

To determine the intracellular localization of E_2 induced pAkt and pERK 1/2 activation within the same cell, twelve randomly selected cortical neurons for each condition were analyzed (Fig 5B). E_2-dependent pAkt activation was localized to the nuclear zone but also apparent throughout the cytoplasm. It is important to note that E_2 induced pAkt localization changed in time-dependent manner. At 10' and 30' pAkt was localized in the perinuclear zone and dendritic areas. At 20' the pAkt signal was diminished and exclusively localized to the soma. The immunocytochemical profile showing an intense distributed pAkt signal at 10' and 30' and a diminished pAkt signal at 20', is consistent with the on-off pattern detected by Western Blot.

E2 activation of ERK 1/2 had different kinetics and localization from pAkt (Fig 5B). The pERK 1/2 intensity was gradual from 10' to 30'with a time-dependent increase. Additionally, as other studies reported (Nilsen and Brinton, 2003a), E_2 induced pERK translocation to the nucleus. In fact, pERK 1/2 was translocated into the nucleus at10' which persisted at 20' and 30'. Further, pERK 1/2 was widely distributed throughout the neuritic extensions branches, with maximal intensity at 30'. At 30', E_2 induced a maximal intensity and intracellular distribution of both pAkt and pERK 1/2 with an overlapping and elevated fluorescence signal. These immunocytochemical data are consistent with results of Western blot analyses (see Fig. 2A-B).

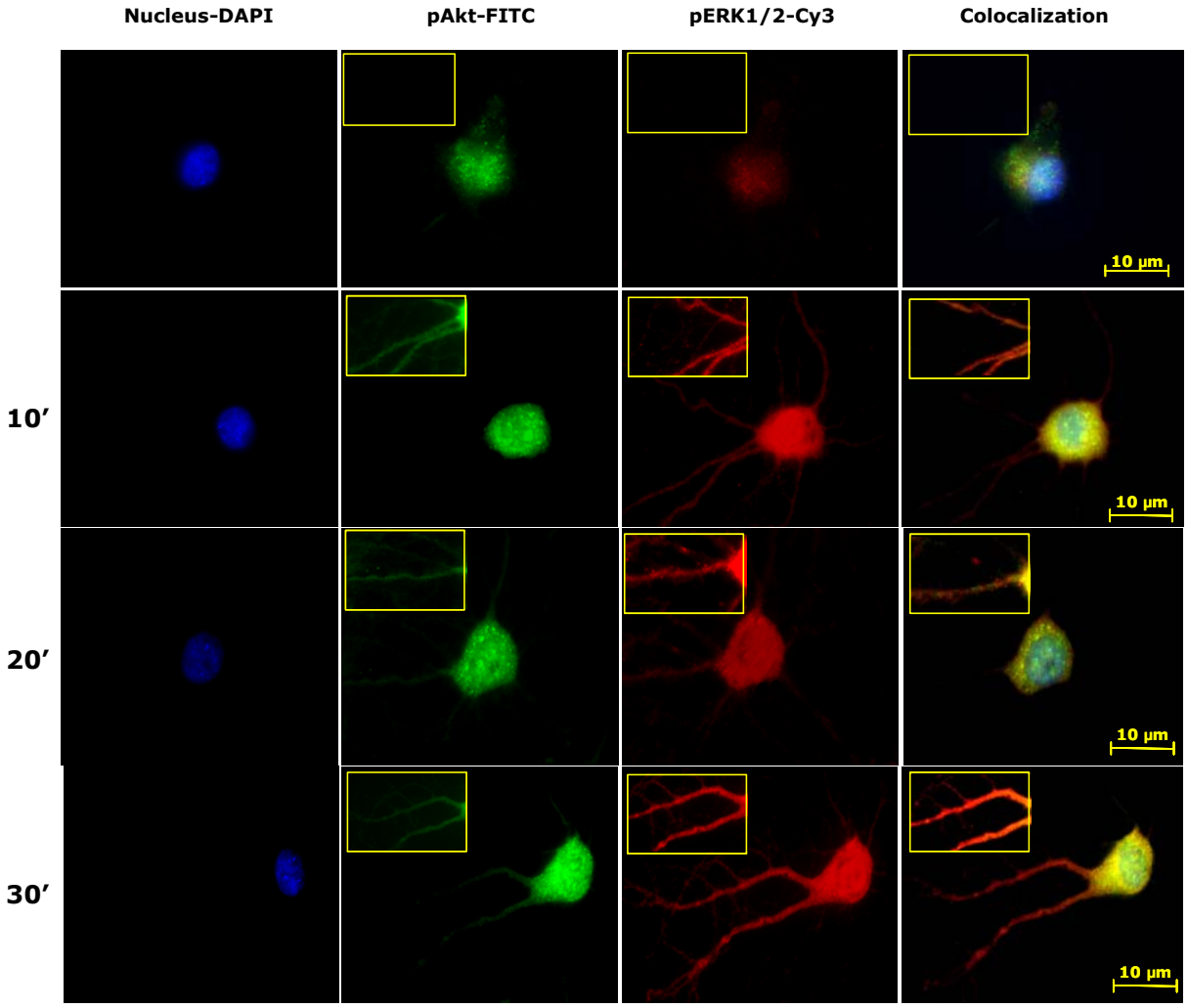


Figure 5B. E_2 induces pAkt and pERK 1/2 translocation to the nucleus. Rat cortical neuron cells were exposed to E_2 (10 nM) at different time points (0',5', 10', 20', 30' and 60'). pERK 1/2 (red) pAkt (green) pAkt/pERK 1/2 colocalization (yellow) immunofluorescence and DAPI nuclear counterstain (blue). E_2 activation induces pAkt and pERK 1/2 translocation within the cell. Fluorescent microscopic images show morphology and relative density of pAkt and pERK 1/2 positive cells in the E_2 treated group versus control (time 0'). Insets are higher magnification

images to highlight pAkt and pERK 1/2 localization in neuronal processes. Data shown are from a single experiment and are representative of 3 independent experiments.

DISCUSSION

Data presented herein demonstrate that E_2 activation of PI3K is the initiation mechanism for E_2 activation of two membrane associated signaling cascade, pAkt and pERK 1/2. Our finding indicate that E_2 activation of PI3K is mediated by a protein-protein interaction between ER and the regulatory protein of PI3K, p85. The ER/p85 protein-protein interaction leads to Akt and ERK 1/2 activation in the same cortical neurons. I E_2 rapidly activated Akt downstream of PI3K, and induced ERK 1/2 downstream of MAPK in neurons in a time-dependent manner. Akt activation was apparent following 10' of E_2 exposure and maximal at 30'. A similar time-course of activation was observed for E2 activation of ERK 1/2 activation, which was evident at 5-10' and maximal at 30'. Quantitative immunocytochemical signal analysis indicated that E_2 activation of both Akt and ERK 1/2 occurred in the same population of cortical neurons.

Our finding of a protein-protein interaction between ER and p85 is consistent with findings of Simoncini and colleagues (Simoncini et al., 2000). These investigators found that ER interacts with p85 in endothelial cells in a ligand-dependent manner and is required for E_2 activation of Akt. Interestingly, the kinetics of E_2 activation of pAkt in endothelial cells was slower than that found in our study of cortical neurons (Simoncini et al., 2000).

The role of PI3K in E_2 signaling has been well established in neurons.(Escobedo et al., 1991; Honda et al., 2001; Singh, 2001; Zhang et al., 2001; Cardona-Gomez et al., 2002) E_2 activation of PI3K/Akt pathway is required for protection against glutamate and β-amyloid induced neurotoxicity (Honda et al., 2000; Singh, 2001). One of the mechanisms by which Akt imparts protection against toxic insults is through phosphorylation of the pro-apoptotic protein BAD, preventing its coupling with Bcl-2 (Datta et al., 1997).

Recent results from our group demonstrated that activation of L-type calcium channels was an upstream requirement for E2 activation of the Src/MAPK signaling pathway (Wu et al., 2005).Requirement of PI3K for activation of L-type calcium channels has been found to mediate IGF-1 and angiotensin II induced activation of L-type Ca^{2+} channels in neurons and myocytes respectively (Blair and Marshall, 1997; Quignard et al., 2001; Sanna et al., 2002). Interestingly, IGF receptor-mediated neuron survival required PI3K/Akt-mediated L-type Ca^{2+} channels activation (Blair et al., 1999). Previous studies from our group demonstrated that in hippocampal neurons E_2 induced Ca^{2+} influx, leading to a rise in both dendritic and nuclear Ca^{2+}(Zhao, 2005). The rise in nuclear Ca^{2+} led to CREB activation (Zhao, 2005). We further found that E_2 induced Ca^{2+} influx was via L-type Ca^{2+} channels which were required for downstream activation of Src/ERK/CREB signaling pathway and BCL-2 expression (Wu et al., 2005). Our working hypothesis is that E2-induced activation of L-type Ca^{2+} channels is through PI3K activation of protein kinase C (PKC). Our laboratory and others have demonstrated an E_2 time dependent interaction between PKC, Ca^{2+} influx and ERK 1/2 activation (Casabona, 1997; Cordey et al., 2003; Setalo et al., 2005; Wu et al., 2005). PI3K activation of PKC is well documented through phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Wymann and Pirola, 1998) and diacylglicerol (DAG) (Standaert et al., 1996) as well as Akt/protein kinase B (PKB).

Several laboratories, using multiple model systems, have observed PI3K activation of MAPK (Perkinton et al., 2002) or an indirect activation via Akt/Raf-1/Mek (Zimmermann and Moelling, 1999). For example, PI3K activity is required for *in vitro* ERK activation in AMPA and NMDA glutamate receptor-mediated signaling in striatal cultures (Perkinton and Sihra, 1998; Perkinton et al., 1999) and for the nerve growth factor-induced sustained activation of ERK during PC12 differentiation (York et al., 2000). Furthermore, PI3K activation was found to

occur upstream of ERK activation in a rat model for associative learning (Lin et al., 2001). Our findings of a E2 activation of PI3K inducible pERK1/2 are consistent with similar signaling strategies in brain and other tissues in which PI3K is required for downstream activation of pERK1/2.

The case for PI3K and E2-inducible pERK1/2 at 30' is more complex. While E_2 activation of pERK 1/2 is PI3K dependent at 10', it appears to be PI3K independent at 30'. Inhibitors of PI3K, completely blocked E_2-induced ERK1/2 activation at 10' yet did not inhibit E2-inducible pERK 1/2 at 30'. The coupling of ER/p85 protein at 10' and the rise of pERK1/2 which is blocked in the presence of PI3K inhibitors at 10' are all consistent with a requirement of PI3K for downstream activation of ERK1/2 at the early time point. Subsequent to this early response when ER/p85 protein are uncoupled, a PI3K independent E2 inducible pERK1/2 is manifested. It is important to note that wortmannin inhibits PI3K activity, binding more deeply in the ATP binding pocket of PI3K catalytic subunit than ATP (Walker et al., 2000) . This action has several interactions in common with the ATP/PI3K complex. In fact, wortmannin effectively competes with PtdIns(4,5)-P2 binding (Wymann et al., 1996) enzyme complex trough a covalent bind which irreversibly inhibits the catalytic subunit, p110 without modifying the p85 structure (Ui et al., 1995). It remains to be determined whether this finding of our paper is an artifact of blocking an E2 preferred signaling pathway or whether the conditions of these experiments unmasked a bona fide alternate route of E2 activation of pERK1/2. One such alternative route may be ER-X, a plasma membrane-associated ER (Toran-Allerand et al., 2002). ER-X is a functional plasma membrane ER, which is enriched in caveolar-like microdomains. Association with caveolar-like microdomain complexes could position ER-X interact with kinases of the MAPK cascade (Toran-Allerand et al., 2002).

PI3K activation of Akt was biphasic in cortical neurons. The biphasic activation pattern was consistent with the coupling and uncoupling of ER and p85 proteins. A direct relationship between PI3K activation and Akt activation was evident at both 10' and 30' as inhibition of PI3K completely blocked E2-induced pAkt at both points.

Results of double labeling immunocytochemical analyses indicated an increase in the number of neurons that expressed Akt and pERK1/2. Moreover, colocalization within the same neurons showed a comparable rise over time. Co-localization of both the pERK1/2 and pAkt was greatest at 30', when activation of these signaling pathways was also maximal. At 30', 68% of cortical neurons exhibited expression of both pAkt and pERK 1/2. This finding suggests that regardless of the complexity of activation, PI3K activation of these two signaling pathways and their cellular localization are tightly coupled in cortical neurons.

In conclusion, we sought to determine whether a single mechanism could serve as a unifying mechanism for E2 activation of Akt and MAPK, two signaling cascades demonstrated to be required for estrogen-inducible neuroprotection against degenerative insults (McEwen, 1992; Toran-Allerand et al., 1999; Brinton, 2001; Nilsen et al., 2002; Yi et al., 2005). Results of this investigation demonstrate that a protein/ protein interaction between estrogen receptor and the regulatory subunit, p85, of PI3K leads to activation of both the Akt and MAPK signaling pathways in cortical neurons. Moreover, E2 activation of Akt and MAPK occurred in the same cells via a PI3K dependent mechanism. Functionally, activation of Akt and MAPK provides a coordinated response that results in inactivation of the proapoptotic protein Bad and activation of antiapoptotic proteins Bcl2 and Bclx (Pike, 1999; Singh, 2001; Nilsen and Brinton, 2003b; Simpkins et al., 2005). Moreover, E2 activation of ERK dependent responses associated with memory function, such as morphogenesis, CREB activation and LTP, creates a coordinated

response network that promotes survival of neurons while simultaneously promoting their function and neural network integration. From a translational perspective, activation of PI3K, Akt and MAPK signaling cascades in neurons could serve as a first in vitro requirement for further development of therapeutics that promote estrogen responses in brain.

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