

Published in final edited form as:

J Neurochem. 2007 April ; 101(1): 232–240. doi:10.1111/j.1471-4159.2006.04360.x.

17- β -Estradiol-mediated activation of extracellular-signal regulated kinase, phosphatidylinositol 3-kinase/protein kinase B-Akt and *N*-methyl-D-aspartate receptor phosphorylation in cortical synaptoneuroosomes

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Abstract

In addition to its well-known activational mechanism, the steroid hormone 17- β -estradiol (E2) has been shown to rapidly activate various signal transduction pathways that could participate in estrogen-mediated regulation of synaptic plasticity. Although the mechanisms underlying these effects are not clearly understood, it has been repeatedly suggested that they involve a plasma membrane receptor which has direct links to several intracellular signaling cascades. To further address the question of whether E2 acts directly at the synapse and through membrane-bound receptors, we studied the effects of E2 and of ligands of estrogen receptors on various signaling pathways in cortical synaptoneuroosomes. Our results demonstrate that E2 elicits *N*-methyl-D-aspartate receptor phosphorylation and activates the extracellular signal-regulated kinase and the phosphatidylinositol 3-kinase/Akt signal transduction pathways in this cortical membrane preparation. Furthermore, we provide evidence for the presence of a membrane-bound estrogen receptor responsible for these effects in cortical synaptoneuroosomes. Our study demonstrates that E2 directly acts at cortical synapses, and that synaptoneuroosomes provide a useful system to investigate the mechanisms by which E2 regulates synaptic transmission and plasticity.

Keywords

estrogen; *N*-methyl-D-aspartate; phosphorylation; plasticity; signaling pathways; synaptoneurosome

17- β -Estradiol (E2) is a steroid hormone that mediates its effects in different target tissues through interaction with estrogen receptors. Physiologically, E2 is involved in sexual differentiation and reproductive behavior, bone formation, and cardiovascular system regulation; moreover, clinical studies in humans suggest that E2 is also implicated in the regulation of cognitive functions as well as in a number of neurological diseases (McEwen and Alves 1999; Gibbs and Gabor 2003). These studies have also been corroborated in laboratory animals (Luine *et al.* 2003). The detailed mechanism by which E2 modulates cognitive functions is still not completely understood, although it has generally been proposed that this effect requires a dual mode of cellular regulation, one involving signaling cascades downstream of a membrane-bound receptor and the other involving regulation of gene expression.

Evidence accumulated over the past several years has demonstrated that E2 rapidly modulates neuronal excitability (Foy and Teyler 1983; Nabekura *et al.* 1986; Gu and Moss 1996), an effect that occurs via activation of intracellular signaling cascades (Singh *et al.* 1999; Bi *et al.* 2000, 2003; Honda *et al.* 2000). These rapid effects of E2, which occur too fast to be mediated by the traditional activational mechanism, are generally assumed to be initiated at the cell membrane and due to the existence of specific membrane-associated estrogen receptors. The identity of the putative estrogen membrane receptors is still unknown, although immunohistochemical analysis suggests that they resemble traditional E2 receptor alpha (ER α) receptors (Evinger and Levin 2005), and biochemical and pharmacological experiments indicate they could also belong to the G-protein-coupled receptor family (Revankar *et al.* 2005; Thomas *et al.* 2005). Moreover, E2 binding to membrane-localized receptors has been shown to activate multiple intracellular cascades, including the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphatidylinositol 3-kinase (PI3-K)/Akt pathways (Kow and Pfaff 2004; Marino *et al.* 2005; Vasudevan *et al.* 2005), and these effects might be responsible for E2-mediated regulation of plasticity and neuroprotection.

The MAPK/ERK pathway occupies a central place in the regulation of synaptic plasticity (Sweatt 2001). Pharmacological manipulations directed at blocking this pathway have consistently produced impairments in synaptic plasticity and in learning and memory; in addition, this pathway is activated by long-term potentiation (LTP)-inducing protocols or in different learning paradigms (Brambilla *et al.* 1997; Berman *et al.* 1998; Blum *et al.* 1999). E2-mediated activation of this pathway could therefore account for the previously reported effects of both exogenous and endogenous E2 on LTP (Kim *et al.* 2005; Smith and McMahon 2005). Similarly, the PI3-K/Akt has also been implicated in the regulation of synaptic plasticity and LTP, and it has been reported that E2 mediates its action via this pathway (Crossthwaite *et al.* 2004; Mendez *et al.* 2005). We previously proposed the hypothesis that E2 activation of either Src tyrosine kinases or ERK results in tyrosine phosphorylation of *N*-methyl-D-aspartate (NMDA) receptors (Bi *et al.* 2000, 2003), and thereby in an increased function of the receptor, an effect that could account for estrogen-mediated LTP enhancement. More recently, it has been proposed that these rapid membrane events could involve activation of metabo-tropic glutamate receptors (Boulware *et al.* 2005).

In the present study, we used cortical synaptoneurosomes as a model to investigate the links between E2 and intracellular signaling pathways. Synaptoneurosomes are functional elements formed from resealed pre- and post-synaptic compartments that maintain most of the pre- and post-synaptic machinery related to transmitter release, receptor and second messenger systems, and have been widely used to study changes in synapses that may occur in response to pharmacological or electrophysiological manipulations (Cohen *et al.* 1977; Iredale *et al.* 1993; Wyszynski *et al.* 1997). Our results strengthen the notion that a membrane-bound estrogen receptor is located at cortical synapses and that its activation regulates NMDA receptor phosphorylation and various signaling pathways.

Materials and methods

Animals

Animals were treated in accordance to the principles and procedures of the National Institutes of Health Guide for the Care and Use of Laboratory Animals; all protocols were approved by the Institutional Animal Care and Use Committee of the University of Southern California. Timed pregnant Sprague–Dawley rats were obtained from Harlan (San Diego, CA, USA) and kept in the vivarium until delivery, in a temperature- and light-controlled environment with a 12 h light/dark cycle. On the day of experimentation, juvenile or adult rats were removed from their home cage and anesthetized using halothane.

Synaptoneurosome preparation

Synaptoneurosomes were prepared from juvenile (postnatal day 20–24) or adult (3 months) Sprague–Dawley rats following a modified protocol to that described by Hollingsworth *et al.* (1985). Following halothane (Sigma, St Louis, MO, USA) anesthesia, rats were decapitated and brains were removed from the cranium and placed into chilled modified Krebs solution buffered with NaHCO₃ (mKrebs/NaHCO₃) (118.5 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH₂PO₄, 10 mmol/L *D*-glucose, 24.9 mmol/L NaHCO₃; pH 7.3) and equilibrated with O₂/CO₂ (95 : 5). Brains were placed into equilibrated and chilled mKrebs/NaHCO₃ containing low calcium and high magnesium (1 mmol/L CaCl₂ and 1.5 mmol/L MgSO₄), and the cortex was dissected out and homogenized in a 7-mL Kontes tissue dounce homogenizer in mKrebs buffered with HEPES (20 mmol/L) (mKrebs/HEPES; 1 mmol/L CaCl₂ and 1.5 mmol/L MgSO₄; pH 7.3) with five passes. Homogenized tissue was filtered through three layers of 100- μ m nylon mesh filter and the resulting suspension was filtered again through a 5- μ m pore size Acrodisc syringe filter with a Supor membrane (Pall Life Sciences, Ann Arbor, MI, USA). The filtrate was then centrifuged at 1000 *g* for 15 min at 4°C, washed one time and centrifuged again. The pellet was resuspended and adjusted to approximately 250 μ g/mL of protein in mKrebs/HEPES with high calcium (2 mmol/L CaCl₂) and low magnesium (1 mmol/L MgSO₄), and the synaptoneurosome suspension was kept on ice until experimental treatments took place.

Synaptoneurosome treatments

Prior to hormone or drug treatments, synaptoneurosomes were pre-incubated at 32°C for 5 min. E2 (Calbiochem, San Diego, CA, USA) was freshly prepared in 80% ethanol (0.174 mol/L); the final ethanol concentration used during experimentation was at or below 0.217 mmol/L. Activation of the ERK pathway was studied by pre-treating (20 min) synaptoneurosomes in the presence or absence of the E2 receptor antagonist 7 α ,17- β -[9[(4,4,5,5,5-pentafluoropentyl) sulfinyl]nonyl] estra-1,3,5(10)-triene-3,17-diol (ICI 182 780; 1 μ mol/L; Tocris Cookson, Ballwin, MO, USA) or the ER α agonist propylpyrazole triol (10 nmol/L; PPT) or E2 receptor beta (ER β) agonist 2,3-bis(4-hydroxyphenyl) propionitrile (10 nmol/L; DPN) (generous gifts from Dr R. Brinton, USC). Membrane-impermeable estrogen (β -estradiol, 6-[*O*-carboxymethyl]oxime: bovine serum albumin (BSA); E2–BSA; Sigma) was prepared using an ultracentrifugation protocol to reduce the amount of free E2 (Taguchi *et al.* 2004). The MEK inhibitors, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059; 50 μ mol/L; Tocris Cookson) or 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126; 10 μ M; Tocris Cookson) were also used to study E2 action on the MAPK/ERK cascade. The PI3-K/Akt pathway inhibitors 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002; 10 μ mol/L; Tocris Cookson) and Wortmannin (100 nmol/L; Tocris Cookson) were used to study the effects of E2 on this signaling cascade. At the end of experimental treatment with hormone or drugs, synaptoneurosomes were centrifuged at 5000 *g* for 5 min at 4°C and the pellet was resuspended in chilled sonication buffer [10 mmol/L Tris–HCl, 0.32 mol/L Sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L NaF, 10 mmol/L Na₃VO₄, and Protease Inhibitor Cocktail (2 mmol/L 4-(2-aminoethyl) benzene-sulphonyl fluoride, 130 μ mol/L Bestatin, 14 μ mol/L E-64, 1 μ mol/L Leupeptin, 0.3 μ mol/L Aprotinin; Sigma), pH 7.3] and sonicated for 10 s. Aliquots of homogenates were used for protein determination by the bicinchoninic acid method (Pierce, Rockford, IL, USA). The remaining homogenates were diluted in Laemmli buffer with 5% mercaptoethanol (Bio-Rad Laboratories, Hercules, CA, USA), boiled for 5 min, and processed for western blotting.

Western blot analysis

For western blot analysis, 10 μ g of proteins were loaded onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Precision unlabeled molecular weight markers and

Strep Tactin-horseradish peroxidase (Bio-Rad Laboratories) were used to estimate apparent molecular weights. The gels were then electroblotted onto Immobilon-P (Millipore Corporation, Bedford, MA, USA). Immunodetection of diphosphorylated ERK1/2 (dpERK; 42 and 44 kDa) was performed by first blocking the membrane with 5% non-fat milk in borate saline (pH 7.6) for 30 min at 22°C, followed by the addition of the primary antibody, anti-diphospho-ERK1/2 (pThr202/pTyr204; 1 : 1000; Cell Signaling Technology, Danvers, MA, USA); the blots were then allowed to react overnight at 22°C in sealed bags. Immunodetection of phosphorylated Akt (pAkt; 60 kDa) was performed using a polyclonal anti-phospho-Akt antibody (pTyr308; 1 : 1000; R&D Systems, Minneapolis, MN, USA). Immunodetection of phosphorylated *N*-methyl-D-aspartate receptor subtype 2B (pNR2B; 180 kDa) subunit was performed using a polyclonal anti-phospho-NR2B antibody (pTyr1472; 1 : 1000; Chemicon, Temecula, CA, USA). Antibody binding to protein was detected using a secondary goat anti-rabbit horseradish peroxidase-labeled antibody (Amersham Biosciences, Piscataway, NJ, USA) at a 1 : 2000 dilution for 1 h at 22°C and visualized autoradiographically on Hyperfilm (Amersham Biosciences) using enzyme-linked chemiluminescence (Pierce) according to the manufacturer's instructions. Blots were stripped (2% sodium dodecyl sulfate, 100 mmol/L mercaptoethanol, and 62.5 mmol/L Tris-HCl, pH 6.7), blocked overnight in 5% non-fat milk in borate saline (pH 7.6), and reprobed for total ERK (ERK; 42 and 44 kDa; 1 : 1000; Cell Signaling Technology), total Akt (60 kDa; 1 : 1000; R&D Systems), or NR2B (180 kDa; 1 : 1000; Chemicon), using a goat anti-rabbit peroxidase-conjugated secondary antibody or a sheep anti-mouse peroxidase-conjugated secondary antibody (Amersham Biosciences).

Densitometric analyses

To quantify protein levels, films were scanned (CanoScan N656U scanner; Canon, Lake Success, NY, USA) and analyzed using a grayscale at 600 dpi. Band densities were calculated using Scion Image software (Scion Corporation, Frederick, MD, USA). Total band intensity values were calculated by subtracting the background for each film, to account for any variation in background intensity across films. Optical density (OD) values were converted to ratios of OD for phosphorylated (OD_{phosphor}) proteins over the corresponding OD for total (OD_{total}) protein; ratios were then normalized by dividing them by the mean ratio value for the vehicle control calculated across independent experiments [$(OD_{\text{phosphor}}/OD_{\text{total}})_{\text{individual values}} \times 1/(OD_{\text{phosphor}}/OD_{\text{total}})_{\text{vehicle average value}}$], to eliminate variation across experiments. Data were then expressed as means \pm SEM for the indicated number of experiments.

Statistical analysis

Significant interactions between vehicle control and E2 treatment were assessed with a two-tailed Student's unpaired *t*-test, when applicable. Two-way and one-way analyses of variance (ANOVA) followed by a Tukey's *post hoc* test were used for multiple comparisons between experimental treatments. Data were analyzed using Analyze-it software (Analyze-it Ltd, Leeds, UK) and significance level was set at 0.05.

Results

E2 rapidly activates ERK in cortical synaptoneurosomes

Treatment of cortical synaptoneurosomes with 10 nmol/L E2 induced ERK phosphorylation (dpERK; pThr202/pTyr204) in a rapid and transient manner in synaptoneurosomes from juvenile and adult rat cortex (Fig. 1). A two-way ANOVA, comparing age and time, did not find any significant interaction between the two factors ($F_{5,24} = 0.67$, $p = 0.648$) nor did it reveal an effect of age ($F_{1,24} = 0.03$, $p = 0.87$); however, it revealed an effect of time of E2 exposure ($F_{5,24} = 8.45$, $p < 0.0001$). This effect of time was observed to begin within 5 min

of exposure to E2 and peaked at 15 min in synaptoneurosomes from juvenile rats (Tukey's *post hoc* test, $p < 0.05$; $n = 3$) and at 30 min in those from adults (Tukey's *post hoc* test, $p < 0.05$; $n = 3$); phosphorylation returned towards basal levels in juvenile and adult rats within 1 h of treatment with the hormone. Increasing E2 concentrations (10 pmol/L–1000 nmol/L E2) increased ERK phosphorylation in cortical synaptoneurosomes from juvenile animals and adult animals (not shown); the effect was maximal at a concentration of 10 nmol/L (Tukey's *post hoc* test, $p < 0.01$; $n = 3$) and remained significant for the higher concentrations of the hormone (Tukey's *post hoc* test, $p < 0.05$; $n = 3$) (Fig. 2). Inhibition of the upstream ERK kinase (Mek) prevented E2-mediated increase in ERK phosphorylation (Fig. 3). The Mek1 inhibitor PD98059 (50 $\mu\text{mol/L}$) (Tukey's *post hoc* test, $p < 0.05$; $n = 4$) and the Mek1/2 inhibitor U0126 (10 $\mu\text{mol/L}$) (Tukey's *post hoc* test, $p < 0.05$; $n = 4$) blocked E2-mediated increase in ERK phosphorylation when compared to E2 treatment. Treatment with E2 and ICI 182 780 uncovered a significant interaction between the hormone and the receptor antagonist (Fig. 4); pre-treatment (20 min) with the E2 receptor blocker, ICI 182 780 (1 $\mu\text{mol/L}$), also blocked E2-mediated ERK phosphorylation (Tukey's *post hoc* test, $p < 0.05$; $n = 3$), while ICI 182 780 alone significantly activated ERK phosphorylation above basal level (Tukey's *post hoc* test, $p < 0.01$; $n = 3$). Both E2 alone and ICI 182 780 alone significantly activated ERK phosphorylation above the level of E2 and ICI 182 780 together (Tukey's *post hoc* test, $p < 0.05$; $n = 3$). On the other hand, treatment with ICI 182 780 and E2 did not block E2-mediated ERK phosphorylation (data not shown). Similar findings have been reported for E2-induced ERK phosphorylation in cortical explant cultures and immortal cell lines (Watters *et al.* 1997; Singer *et al.* 1999; Singh *et al.* 1999; Belcher *et al.* 2005). In order to determine whether interaction of E2 with ER α or ER β was responsible for ERK activation, we tested the effects of ligands specific for these subtypes of estrogen receptors (ER) in cortical synaptoneurosomes (Fig. 5a). Treatment with E2 and the two receptor agonists significantly induced ERK activation. Both PPT (Tukey's *post hoc* test, $p < 0.001$, $n = 3$), an ER α agonist, and DPN (Tukey's *post hoc* test, $p < 0.01$, $n = 3$), an ER β agonist, at 10 nmol/L increased ERK phosphorylation when incubated for 30 min with cortical synaptoneurosomes.

E2 mediates its effects in synaptoneurosomes by interaction with a membrane receptor

It is possible that E2 requires binding to a membrane-bound receptor in order to elicit rapid activation of ERK in synaptoneurosomes. To test this hypothesis, we incubated cortical synaptoneurosomes with a proposed membrane-impermeable form of estrogen (E2-BSA; Fig. 5b); under this condition, we also observed a significant activation of ERK phosphorylation. Incubation with 100 nmol/L E2-BSA (Tukey's *post hoc* test, $p < 0.05$; $n = 3$) significantly activated ERK after 30 min of treatment, suggesting the presence of a membrane-bound estrogen receptor (Benten *et al.* 2001). We also verified that our synaptoneurosomes preparations contained both estrogen receptors (ER α and ER β) using western blot analysis (data not shown); the antibodies (ER α , MC-20 and H-184; ER β , H-150) that we used for immunodetection of the receptors have previously been reported to label membrane bound receptors expressed in neurons and other cell types (Fiorini *et al.* 2003; Marin *et al.* 2003).

E2 elicits Akt phosphorylation in cortical synaptoneurosomes

Treatment of cortical synaptoneurosomes with E2 also activated Akt as detected by an increase in levels of pAkt (Fig. 6). Quantification of pAkt (pTyr308) levels indicated that treatment of synaptoneurosomes with 10 nmol/L E2 for 30 min resulted in a significant increase in pAkt levels. E2 significantly increased Akt phosphorylation after 30 min (Tukey's *post hoc* test, $p < 0.05$, $n = 4$) and the PI3-K inhibitors LY294002 (10 $\mu\text{mol/L}$) and Wortmannin (100 nmol/L) (Tukey's *post hoc* test, $p < 0.01$ and $p < 0.05$, $n = 4$) blocked E2-mediated Akt phosphorylation. In addition, treatment with Mek inhibitors also had an effect

on Akt phosphorylation; PD98059 (50 $\mu\text{mol/L}$) or U0126 (10 $\mu\text{mol/L}$) blocked E2-mediated increased Akt phosphorylation (Tukey's *post hoc* test, $p < 0.05$, $n = 4$) (Fig. 7). This result contrasts with a previous report indicating that PD98059 did not block E2-mediated increase in Akt phosphorylation in endothelial cells (Simoncini *et al.* 2000). Furthermore, treatment with LY294002 (10 $\mu\text{mol/L}$) and Wortmannin (100 nmol/L) (Fig. 8) block E2-mediated increase in ERK phosphorylation; in the presence of either LY294002 or Wortmannin, E2 failed to significantly increase ERK phosphorylation [Student's unpaired *t*-test indicated a significant difference ($p < 0.0001$) between vehicle and E2 treated synaptoneurosome]. These results suggest the existence of crosstalk between the ERK and Akt pathways.

E2 enhances NMDA receptor tyrosine phosphorylation in cortical synaptoneurosome

We previously reported that E2 treatment of hippocampal slices resulted in increased phosphorylation of NR2 subunits of NMDA receptors (Bi *et al.* 2000). We therefore tested whether E2 treatment of synaptoneurosome could also modify NR2B subunit phosphorylation (Fig. 9). Incubation of synaptoneurosome with 10 nmol/L E2 for 30 min increased levels of phosphorylated NR2B (pNR2B; pTyr1472) subunit by 38% (Tukey's *post hoc* test, $p < 0.05$, $n = 3$). Treatment with U0126 (10 $\mu\text{mol/L}$) prevented E2-mediated increase in pNR2B (Tukey's *post hoc* test, $p < 0.05$, $n = 3$), while treatment with Wortmannin did not (Tukey's *post hoc* test, $p < 0.05$, $n = 3$), suggesting that activation of the ERK pathway but not the PI3-K pathway is involved in NR2B phosphorylation. It has previously been reported that interactions between NR2B and Src kinases are accompanied by ERK and Akt phosphorylation (Yamada and Nabeshima 2003), while others have indicated that NR2B could be directly phosphorylated by *c*-Src (Cheung and Gurd 2001). Whether E2-mediated phosphorylation of NR2B is dependent on Src/NR2B interaction in synaptoneurosome will be addressed in future studies.

Discussion

Our results indicate that E2 rapidly activates several signaling pathways in cortical synaptoneurosome. Previous results have shown that E2 rapidly activates the ERK and PI3-K/Akt signal transduction pathways in cortical neurons (Singh *et al.* 1999; Honda *et al.* 2000) and increases NR2B tyrosine phosphorylation in cultured hippocampal slices (Bi *et al.* 2000). In the present study, we demonstrated that E2 also activates the ERK and Akt pathways and increases NR2B tyrosine phosphorylation in cortical synaptoneurosome. Our present results further indicate that E2 acts at the level of the synapse to activate various intracellular signaling pathways.

It has been repeatedly proposed that the effects of E2 on ERK and Akt are mediated through an interaction with a membrane-associated receptor (Marino *et al.* 2005). The nature of this membrane-associated estrogen receptor(s) remains ill-defined (Pappas *et al.* 1995; Qiu *et al.* 2003), and the traditional estrogen receptor antagonists ICI 162,780 and tamoxifen have been found not to block E2-induced rapid ERK activation or act as agonist when present alone (Watters *et al.* 1997; Singh *et al.* 1999; Belcher *et al.* 2005). *In vivo* ERK activation can also be elicited by the non-membrane permeable estrogen receptor ligand, E2-BSA, an effect insensitive to ICI 162,780 and tamoxifen (Kuroki *et al.* 2000). In contrast to these aforementioned findings, other reports have indicated that traditional ER antagonists block E2-mediated increase in ERK phosphorylation in cortical neurons (Singer *et al.* 1999; Yokomaku *et al.* 2003).

Immunohistochemical studies using antibodies directed toward typical ERs (α or β) have shown the presence of estrogen receptors in mitochondria, endoplasmic reticulum, as well as in synaptic membranes (Blaustein *et al.* 1992; Chen *et al.* 2004; Revankar *et al.* 2005; Romeo *et al.* 2005). In these studies, the nature of the membrane-bound estrogen receptor

has been proposed to resemble ER α (Razandi *et al.* 2004; Acconcia *et al.* 2005), and the identity of the ER β localized to membranes remains controversial (Yang *et al.* 2004, 2006). However, it also appears that there may be other novel estrogen receptors (e.g. ER46, GPR30 or ER-X) resembling ERs, which could be involved in the rapid activation of signaling cascades (Li *et al.* 2004; Thomas *et al.* 2005; Toran-Allerand 2005). Recent studies using cell lines expressing or not expressing estrogen receptors have reported conflicting results in terms of receptor pharmacology, identity of the receptor, and binding of receptor antagonists (Nethrapalli *et al.* 2005; Revankar *et al.* 2005; Thomas *et al.* 2005). Western blot analysis of synaptoneurosome proteins using antibodies directed against ER α (MC-20 and H-184) or ER β (H-150) indicated the presence of both types of estrogen receptors in our synaptoneurosome preparations. However, our results indicate that the same uncertainty regarding the nature of the membrane-associated ER receptors is present in synaptoneurosome, as agonists of both ER α and ER β were able to activate ERK.

Despite the ambiguity regarding the nature of the estrogen receptors, interactions of estrogen receptors with tyrosine kinases could account for E2-mediated increase in NR2B phosphorylation. NR2B phosphorylation has been associated with tyrosine kinase activity (Kalluri and Ticku 1999), and E2-induced phosphorylation of NR2B at Tyr1472 could serve as a mechanism to selectively increase NR2B insertion into synaptic membranes and increase NMDA receptor function (Cyr *et al.* 2001; Lavezzari *et al.* 2003; Qiu *et al.* 2003; Besshoh *et al.* 2005). This result fits well with previous results indicating that a Src-family kinase inhibitor prevented E2-mediated increase in hippocampal LTP and tyrosine phosphorylation of NR2 subunits (Bi *et al.* 2000).

The mechanism by which E2 rapidly activates the ERK and Akt pathways is still unclear. E2-mediated activation of second messenger systems, such as Ca²⁺, phosphatidylinositol, and cAMP has been well documented (McEwen and Alves 1999; Wu *et al.* 2005), and it is possible there may be additional mechanisms involved in mediating the hormone action. E2 has been reported to increase Src phosphorylation and to stimulate interactions between ER α and Src to elicit ERK activation (Singer *et al.* 1999; Nethrapalli *et al.* 2001; Song *et al.* 2005), while E2 treatment also initiates interaction between ER α , Src and p85, the regulatory subunit of PI3-K (Simoncini *et al.* 2000; Castoria *et al.* 2001). In addition, activation of both ERK and Akt by E2 can be prevented by Src-family kinase inhibitors (PP1 or PP2), which subsequently lead to the blockade of ERK and Akt phosphorylation (Singer *et al.* 1999; Castoria *et al.* 2001; Haynes *et al.* 2003).

In conclusion, our results indicate that E2 activates several intracellular signaling pathways at the levels of the synapse by stimulating a membrane-associated receptor. These include the ERK pathway as well as the PI3-K/Akt pathway, and are associated with increased phosphorylation of NMDA receptor subunits. Furthermore, activation of these signaling cascades is an indicator that Src kinases are involved in mediating E2 action in synaptic connections. Finally, our results indicate that synaptoneurosome represent a good model system to investigate the molecular mechanisms of E2 action in synaptic transmission and plasticity.

Acknowledgments

This research was supported by Grant AG14751 from the National Institute on Aging (PI: Dr C.E. Finch).

Abbreviations used

BSA bovine serum albumin

dpERK	diphosphorylated extracellular signal-regulated kinase
DPN	2,3-bis(4-hydroxyphenyl) propionitrile
E2	17- β -estradiol
ER	estrogen receptors
ERα	E2 receptor alpha
ERβ	E2 receptor beta
ERK	extracellular signal-regulated kinase
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
Mek	upstream ERK kinase
NMDA	<i>N</i> -methyl-D-aspartate
NR2B	<i>N</i> -methyl-D-aspartate receptor subtype 2B
OD	optical density
p	phosphorylated
PI3-K	phosphatidylinositol 3-kinase
PPT	propylpyrazole triol

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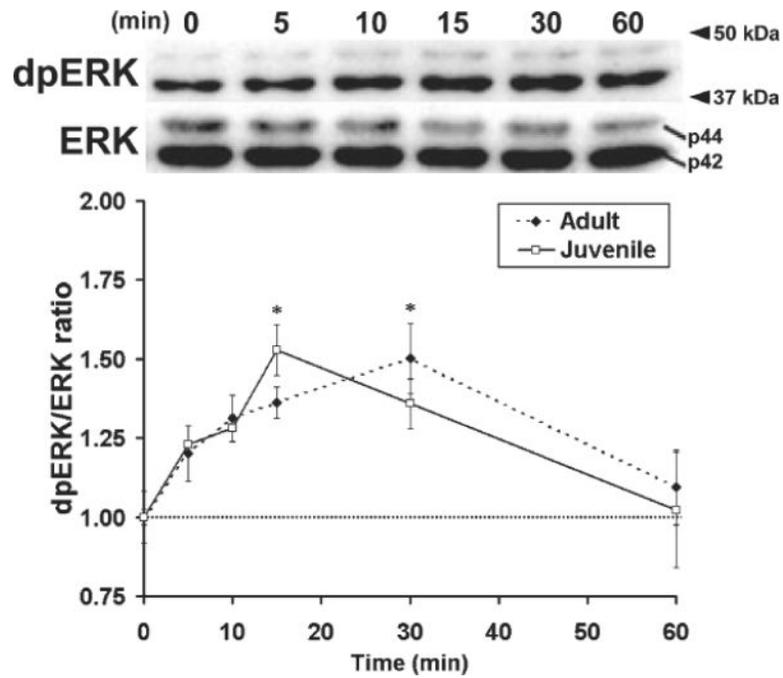


Fig. 1. Effects of 17- β -estradiol on diphosphorylated extracellular signal-regulated kinase 1/2 (dpERK) with increased time of incubation. Cortical synaptoneurosomes were prepared from juvenile (postnatal day 20–24, squares) (one-way ANOVA, $F_{5,12} = 4.956$, $p = 0.011$) or adult (3 months old, diamonds) (one-way ANOVA, $F_{5,12} = 4.162$, $p = 0.02$) rats, and incubated in the absence or presence of 10 nmol/L 17- β -estradiol for the indicated periods of time. Top: representative western blots from juvenile rats labeled with antibodies against dpERK (top) and ERK (bottom) across the six experimental conditions. All blots used for dpERK analysis were stripped and reprobated with ERK, and all experiments were performed in duplicate. Bottom: quantification of the results obtained in three independent experiments. Results are expressed as ratio values (means \pm SEM) for dpERK over ERK (see Materials and methods). Juvenile (Tukey's post hoc test, * $p < 0.05$; 0 vs. 15 min); adult (Tukey's post hoc test, * $p < 0.05$; 0 vs. 30 min).

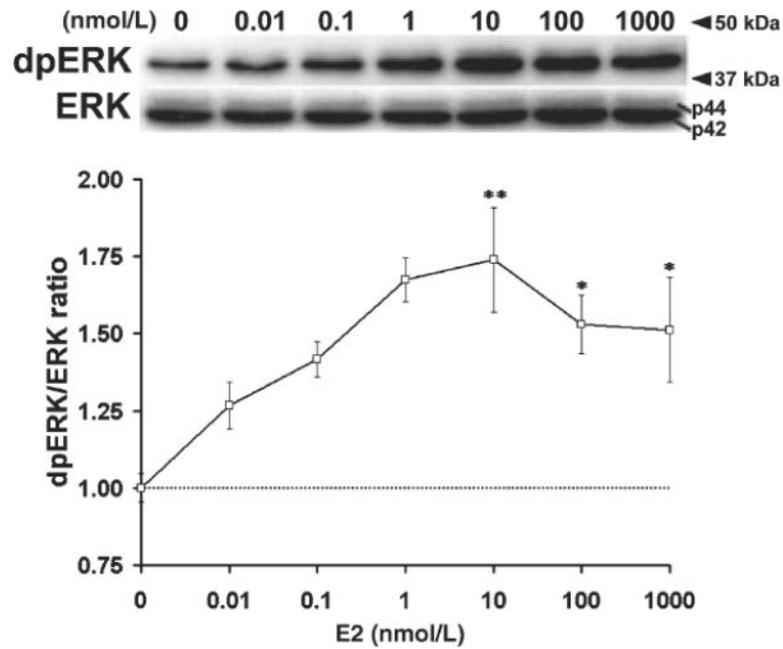


Fig. 2. Effects of various concentrations of 17- β -estradiol (E2) on diphosphorylated extracellular signal-regulated kinase (dpERK) levels in cortical synaptoneurosomes. Synaptoneurosomes from juvenile rats were treated with increasing concentrations (0.01–1000 nmol/L) of E2 for 30 min (one-way ANOVA, $F_{6,14} = 6.908$, $p = 0.001$). Top: representative western blots labeled with antibodies against dpERK (top) and ERK (bottom) across the seven experimental conditions. All blots used for dpERK analysis were stripped and reprobbed with ERK, and all experiments were performed in duplicate. Bottom: quantification of the results obtained in three independent experiments. Results are expressed as ratio values (means \pm SEM) for dpERK over ERK (see Materials and methods) (Tukey's post hoc test, * $p < 0.05$, ** $p < 0.01$; vs. 0 nmol/L).

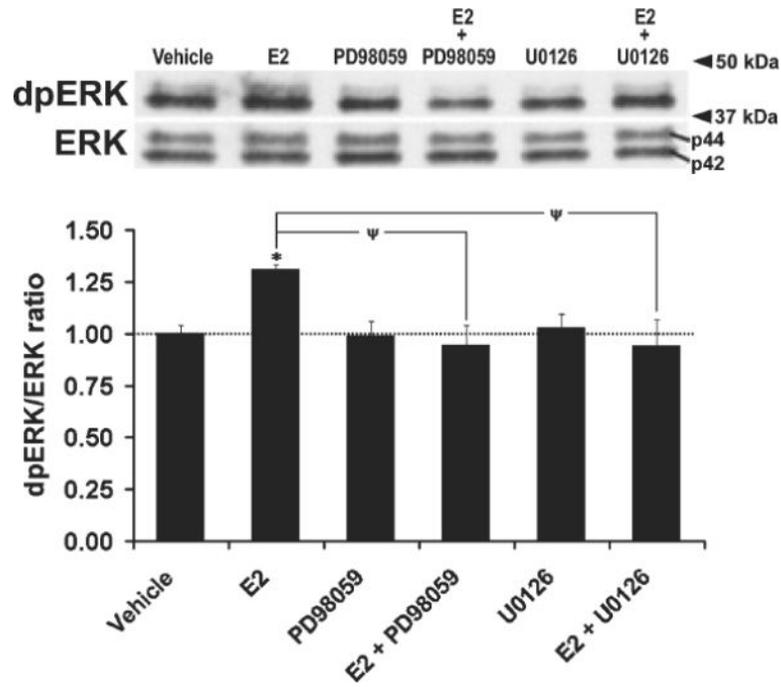


Fig. 3.

Effects of upstream ERK kinase inhibitors on 17- β -estradiol (E2)-induced extracellular signal-regulated kinase (ERK) activation in cortical synaptoneurosomes. Synaptoneurosomes prepared from juvenile rats were incubated for 30 min with 10 nmol/L E2 in the absence or presence of PD98059 (50 μ mol/L) or U0126 (10 μ mol/L) (one-way ANOVA, $F_{5,18} = 2.917$, $p = 0.042$). PD98059 or U0126 was added to the synaptoneurosomes 20 min prior to E2. Top: representative western blots labeled with antibodies against diphosphorylated ERK (dpERK) (top) or ERK (bottom). Bottom: quantification of the results obtained in four independent experiments. All blots used for dpERK analysis were stripped and reprobbed with ERK, and all experiments were performed in duplicate. Results are expressed as ratio values (means \pm SEM) for dpERK over ERK (see Materials and methods) (Tukey's post hoc test, * $p < 0.05$; vs. vehicle) (Tukey's post hoc test, $\Psi p < 0.05$; vs. E2).

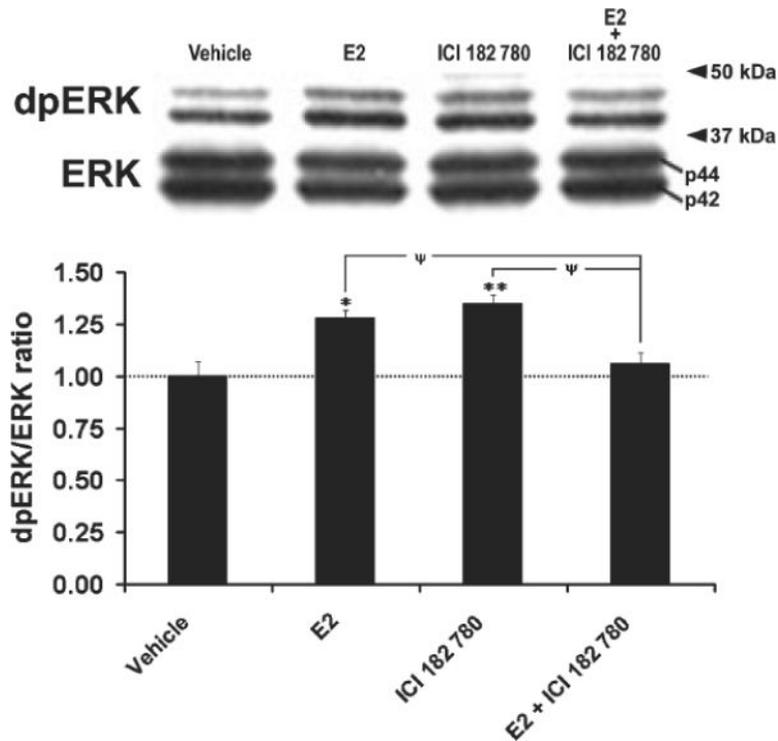


Fig. 4. Effects of the estrogen receptor antagonist ICI 182 780 on 17- β -estradiol (E2)-induced extracellular signal-regulated kinase (ERK) activation in cortical synaptoneuroosomes. Synaptoneuroosomes prepared from juvenile rats were incubated for 30 min with 10 nmol/L in the absence or presence of ICI 182 780 (1 μ mol/L) (one-way ANOVA, $F_{3,8} = 10.23$, $p = 0.004$). ICI 182 780 was added to the synaptoneuroosomes 20 min prior to E2. Top: representative western blots labeled with antibodies against diphosphorylated ERK (dpERK) (top) or ERK (bottom). Bottom: quantification of the results obtained in four independent experiments. All blots used for dpERK analysis were stripped and reprobbed with ERK, and all experiments were performed in duplicate. Results are expressed as ratio values (means \pm SEM) for dpERK over ERK (see Materials and methods) (Tukey's *post hoc* test, $*p < 0.05$, $**p < 0.01$; vs. vehicle) (Tukey's *post hoc* test, $\psi p < 0.05$; vs. 17- β -estradiol).

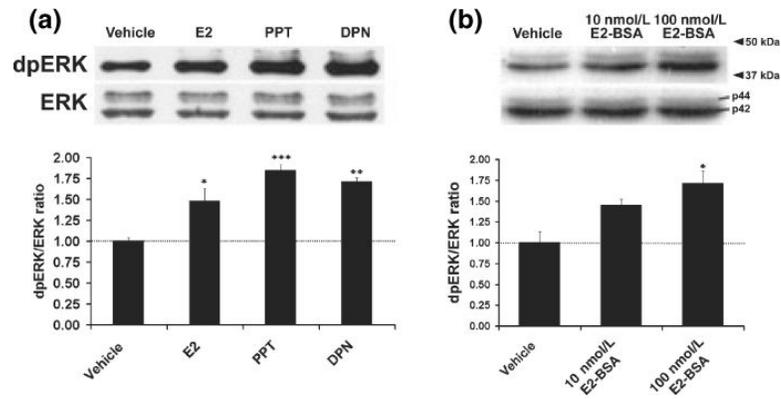


Fig. 5. Effects of estrogen receptors agonists on 17- β -estradiol (E2)-induced extracellular signal-regulated kinase (ERK) activation in cortical synaptoneuroosomes. Synaptoneuroosomes prepared from juvenile rats were incubated for 30 min with (a) 10 nmol/L E2 or propylpyrazole triol (PPT) or 2,3-bis(4-hydroxyphenyl) proprionitrile (DPN) (one-way ANOVA, $F_{3,8} = 16.82$, $p = 0.0008$) and (b) E2-conjugated bovine serum albumin (E2-BSA) (one-way ANOVA, $F_{2,6} = 8.461$, $p = 0.018$). Top: representative western blots labeled with antibodies against diphosphorylated ERK (dpERK, top bands) or ERK (bottom bands). Bottom: quantification of the results obtained in three independent experiments. All blots used for dpERK analysis were stripped and reprobbed with ERK, and all experiments were performed in duplicate. Results are expressed as ratio values (means \pm SEM) for dpERK over ERK (see Materials and methods) (Tukey's *post hoc* test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; vs. vehicle).

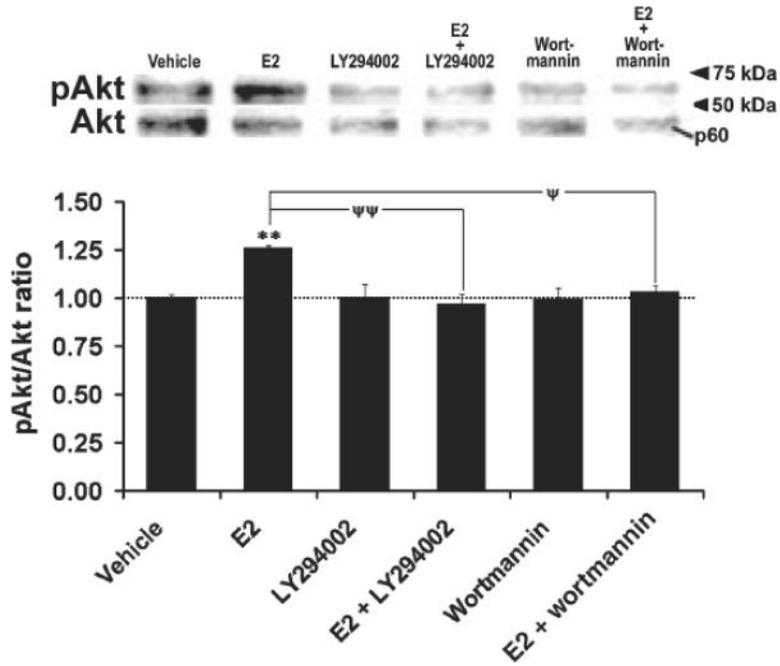


Fig. 6. Activation of the phosphatidylinositol 3-kinase/Akt signal transduction pathway by 17- β -estradiol (E2) in cortical synaptoneurosomes. Synaptoneurosomes prepared from juvenile rats were incubated for 30 min with 10 nmol/L E2 in the absence or presence LY294002 (10 μ mol/L) or Wortmannin (100 μ mol/L) (one-way ANOVA, $F_{5,18} = 4.511$, $p = 0.008$). Inhibitors were added to the synaptoneurosomes 20 min prior to E2. At the end of incubation, aliquots were processed for western blots and labeled with antibodies against phosphorylated Akt (pAkt) or Akt. Top: representative western blots labeled with antibodies against phosphorylated Akt (pAkt) (pTyr308) (top) or Akt (bottom). Bottom: quantification of the results obtained in four independent experiments. All experiments were performed in duplicate. Results are expressed as ratio values (means \pm SEM) for pAkt over Akt (see Materials and methods) (Tukey's *post hoc* test, $**p < 0.01$; vs. vehicle) (Tukey's *post hoc* test, $\Psi p < 0.05$, $\Psi\Psi p < 0.01$; vs. E2).

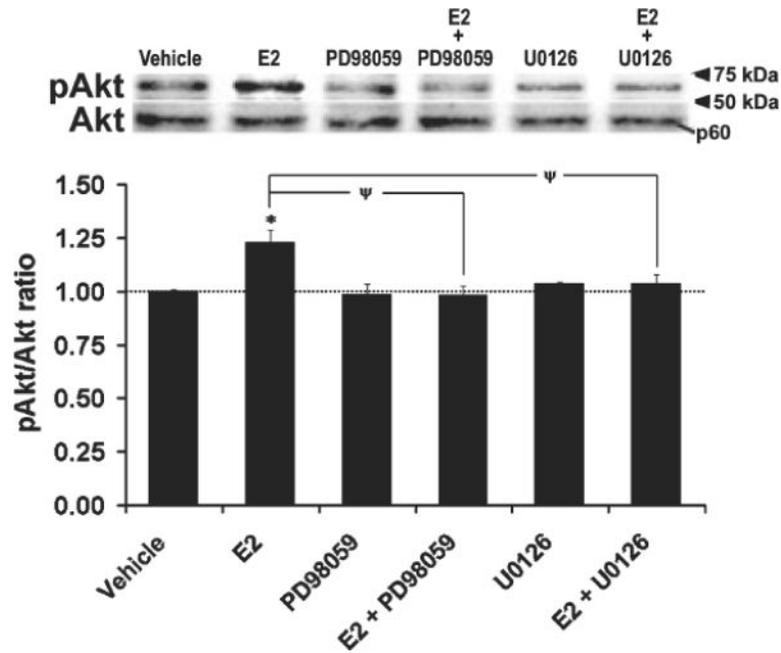


Fig. 7. Effect of Mek inhibitors on 17- β -estradiol (E2)-induced activation of phosphatidylinositol 3-kinase/Akt signaling in cortical synaptoneurosomes. Synaptoneurosomes prepared from juvenile rats were incubated for 30 min with 10 nmol/L E2 following pre-treatment (20 min) with PD98059 (50 μ mol/L) or U0126 (1 μ mol/L) (one-way ANOVA, $F_{5,12} = 5.064$, $p < 0.01$). Top: representative western blots labeled with antibodies against pAkt (top) or Akt (bottom). Bottom: quantification of the results obtained in three independent experiments. All blots used for pAkt analysis were stripped and reprobbed with Akt, and all experiments were performed in duplicate. Results are expressed as ratios values (means \pm SEM) for pAkt over Akt (see Materials and methods) (Tukey's *post hoc* test, * $p < 0.05$; vs. vehicle) (Tukey's *post hoc* test, $\Psi p < 0.05$; vs. E2).

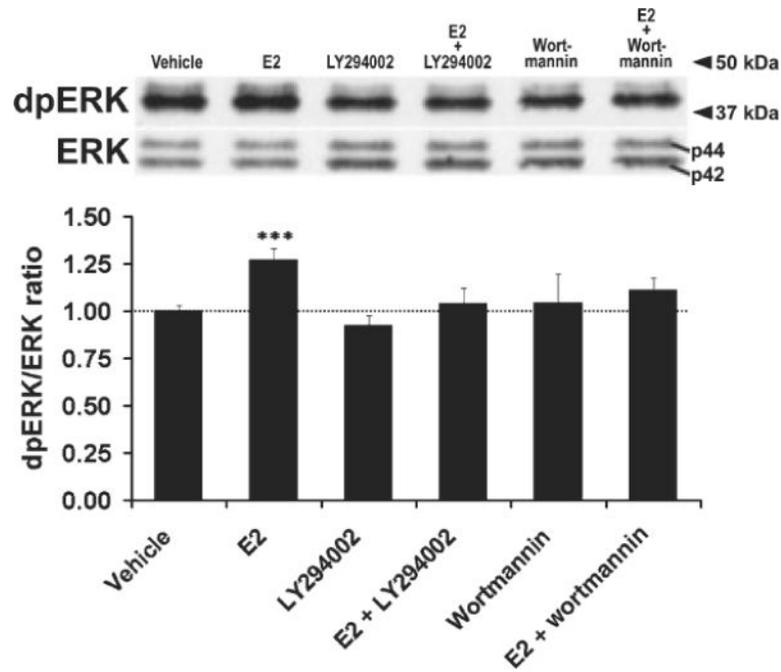


Fig. 8. Effect of phosphatidylinositol 3-kinase/Akt inhibitors on 17- β -estradiol (E2)-mediated extracellular signal-regulated kinase (ERK) phosphorylation. Synaptoneurosomes prepared from juvenile rats were incubated for 30 min with 10 nmol/L E2 following pre-treatment (20 min) with LY294002 (10 μ mol/L) or Wortmannin (100 nmol/L) (one-way ANOVA, $F_{5,18} = 3.265$, $p = 0.028$). Top: representative western blots labeled with antibodies against diphosphorylated ERK (dpERK) (top) or ERK (bottom). Bottom: quantification of the results obtained in three independent experiments. All blots used for dpERK analysis were stripped and reprobbed with ERK, and all experiments were performed in duplicate. Results are expressed as ratio values (means \pm SEM) for dpERK over ERK (see Materials and methods) (two-tailed Student's unpaired t -test, *** $p < 0.0001$; vs. vehicle).

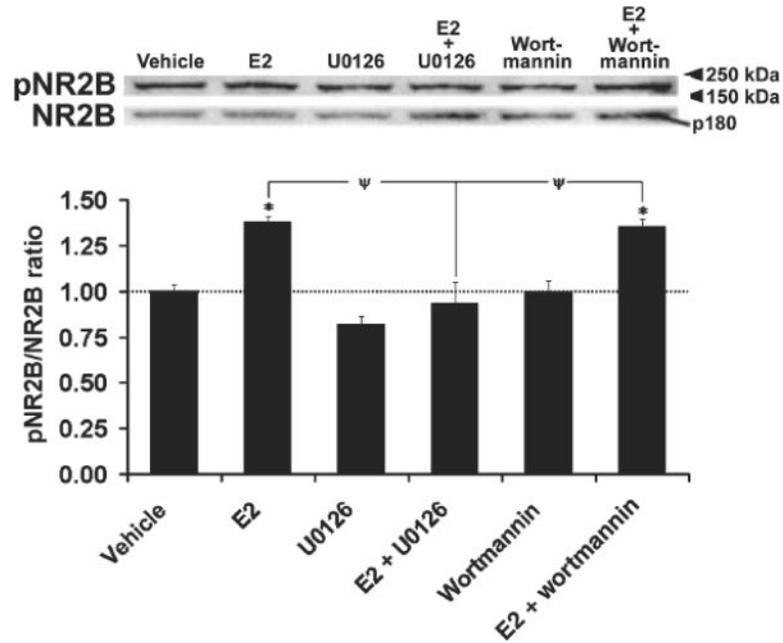


Fig. 9.

Effect of 17- β -estradiol (E2) on phosphorylation of the *N*-methyl-D-aspartate receptor subtype 2B (NR2B) subunit of *N*-methyl-D-aspartate receptors in cortical synaptoneurosomes. Synaptoneurosomes prepared from young rats were incubated for 30 min with 10 nmol/L E2 in the absence or presence of U0126 (1 μ mol/L) or Wortmannin (100 nmol/L) (one-way ANOVA, $F_{5,12} = 12.81$, $p = 0.0002$). Inhibitors were added to the synaptoneurosomes 20 min prior to E2. At the end of incubation, aliquots were processed for western blots labeled with antibodies against phosphorylated NR2B (pNR2B) or NR2B. Top: representative western blots labeled with antibodies against pNR2B or NR2B. Bottom: quantification of the results obtained in three independent experiments. All experiments were performed in duplicate. Results are expressed as ratio values (means \pm SEM) for pNR2B over NR2B (see Materials and methods) (Tukey's *post hoc* test, $*p < 0.05$; vs. vehicle) (Tukey's *post hoc* test, $^{\Psi}p < 0.05$; vs. E2 and E2 + Wortmannin).