Rapid Signaling of Estrogen to WAVE1 and Moesin Controls Neuronal Spine Formation via the Actin Cytoskeleton

Angel Matias Sanchez, Marina Ines Flamini, Xiao-Dong Fu, Paolo Mannella, Maria Silvia Giretti, Lorenzo Goglia, Andrea Riccardo Genazzani, and Tommaso Simoncini

Molecular and Cellular Gynecological Endocrinology Laboratory (A.M.S., M.I.F., P.M., M.S.G., L.G., A.R.G., T.S.), Department of Reproductive Medicine and Child Development, University of Pisa, Pisa 56100, Italy; and Department of Physiology (X.-D.F.), Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China

Estrogens are important regulators of neuronal cell morphology, and this is thought to be critical for gender-specific differences in brain function and dysfunction. Dendritic spine formation is dependent on actin remodeling by the WASP-family verprolin homologous (WAVE1) protein, which controls actin polymerization through the actin-related protein (Arp)-2/3 complex. Emerging evidence indicates that estrogens are effective regulators of the actin cytoskeleton in various cell types via rapid, extranuclear signaling mechanisms. We here show that 17β-estradiol (E2) administration to rat cortical neurons leads to phosphorylation of WAVE1 on the serine residues 310, 397, and 441 and to WAVE1 redistribution toward the cell membrane at sites of dendritic spine formation. WAVE1 phosphorylation is found to be triggered by a Gα_i/Gβ protein-dependent, rapid extranuclear signaling of estrogen receptor α to c-Src and to the small GTPase Rac1. Rac1 recruits the cyclin-dependent kinase (Cdk5) that directly phosphorylates WAVE1 on the three serine residues. After WAVE1 phosphorylation by E2, the Arp-2/3 complex concentrates at sites of spine formation, where it triggers the local reorganization of actin fibers. In parallel, E2 recruits a Gα_{13}-dependent pathway to RhoA and ROCK-2, leading to activation of actin remodeling via the actin-binding protein, moesin. Silencing of WAVE1 or of moesin abrogates the increase in dendritic spines induced by E2 in cortical neurons. In conclusion, our findings indicate that the control of actin polymerization and branching via moesin or WAVE1 is a key function of estrogen receptor α in neurons, which may be particularly relevant for the regulation of dendritic spines. **(*Molecular Endocrinology* 23: 1193–1202, 2009)**

The brain is an important target of sex steroids, which play multiple regulatory roles at this level (1). One of the most intriguing actions of these hormones is the control of brain plasticity, which is critical for memory, learning, and cognition (2). Clinical studies suggest that the lack of estrogens, such as after the menopause, may be related to the progression of degenerative diseases, like Alzheimer's dementia or Parkinson's disease (3), and the hypothesis that estrogen administration to postmenopausal women might decrease the progression of these conditions is lingering (4).

At the basis of brain plasticity is the ability of neurons and glial cells to remodel their mutual connections, which requires major architectural changes of the cytoskeleton and cell membrane (5). These morphological modifications depend on the generation of dynamic structural modifications of the cytoskeleton, via actin polymerization and depolymerization, and on the development of protrusive membrane structures, such as lamellipodia and filopodia. These structures are critical in the generation of cell-cell interconnections and represent the starting platform for cytokinesis, adhesion, and motility (6).

Recent findings indicate that sex steroid hormones are fundamental regulators of cell morphology and motility in human cells and that many of these actions are played via rapid signaling to the actin cytoskeleton achieved via the recruitment of actin-binding proteins, including the Ezrin/Radixin/Moesin (ERM) protein moesin (7–9). In these cells, estrogen or progesterone exposure leads to

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Abbreviations: Arp, Actin-related protein; Cdk5, cyclin-dependent kinase; DPN, 2,3-bis(4-hydroxyphenil)-propionitrile; E2, 17β-estradiol; ERα, estrogen receptor α; IP, immunoprecipitate; PON, phosphorothioate oligonucleotide; PPT, 4,49,40-(4 propyl-[1H]-pyrazole-1,3,5-triyl); PTX, pertussis toxin; siRNA, small interfering RNA.

rapid modifications of the interaction with the surrounding environment and nearby cells (7–9). The control of cell morphology is also enacted by estrogens in the central nervous system, where changes in neuron/neuron interconnections and dendritic spine density ensue related to the cyclical changes in estrogen levels (10).

Between the many regulators of the actin cytoskeleton, the WASP-family verprolin homologous (WAVE) family proteins, neural WASP, WAVE1, and WAVE3 are important in brain cells, where they link extracellular stimuli to actin reorganization (11).

WAVE1 acts as a scaffolding protein that relays signals from small GTPases to the actin-related protein (Arp)-2/3 complex that is responsible for the branching of actin filaments. Loss of WAVE1 *in vivo* or in cultured neurons results in a decrease in mature dendritic spines (12), suggesting that phosphorylation/dephosphorylation of WAVE1 in neurons plays a role in the formation of the filamentous actin cytoskeleton and thus in the regulation of dendritic spine morphology.

The aim of the present study was to explore the molecular basis of the action of estrogen on neuronal morphology. In particular, we wished to identify whether these actions may require the regulation of the actin cytoskeleton via WAVE1 or moesin and to characterize the intracellular cascades that may be recruited during this signaling.

Results

Estrogen induces rapid cytoskeletal and cell membrane remodeling and dendritic spine formation in cortical neurons

As a first step in identifying the effects of estrogen on neuronal morphology, we studied the actions of a rapid estrogen exposure on the actin cytoskeleton. Treatment with 17β-estradiol (E2, 10 nM) resulted in a rapid change in actin organization, with a remodeling of the actin cytoskeleton toward the cell membrane resulting in a thickening of the membrane (Fig. 1, A–C). This phenomenon was time dependent and transient, being maximal after 15–20 min and reversing to baseline after 60 min (Fig. 1, A–C). In parallel, an increased number of dendritic spines was found that followed the same time course (Fig. 1, A and D).

Estrogen rapidly induces WAVE1 and moesin phosphorylation

WAVE1 is regulated by phosphorylation on three serine residues in the proline-rich domain (Ser^{310}, Ser^{397}, and Ser^{441}) (12). Treatment with E2 (10 nM) rapidly increased the phosphorylation of WAVE1 on all three serine residues along a temporal pattern that was consistent with that of actin remodeling (Fig. 2, A and B). In parallel, E2 induced a dynamic phosphorylation on Thr^{558} of the actin-binding protein moesin (Fig. 2C), which is responsible for triggering actin remodeling toward the membrane in endothelial and breast cancer cells (7–9).

Upon exposure to E2, WAVE1 was redistributed toward the plasma membrane, in particular along the dendritic protrusions (Fig. 2D), in parallel with actin and cell membrane remodeling (Fig. 2, E and F).

To test whether actin remodeling by WAVE1 or moesin is required for the estrogen-dependent membrane rearrangement in neurons, we silenced WAVE1 with small interfering RNAs (siRNAs) and moesin with antisense phosphorothioate oligonucleotides (PONs). WAVE1 expression was significantly reduced when neurons were transfected for 48 h with WAVE1 siRNAs (supplemental Fig. A, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). In WAVE1-silenced neurons, E2 failed to induce actin reorganization (Fig. 2, G and H). Moesin silencing resulted in lack of actin remodeling in response to E2, in the absence of any modification of WAVE1 expression or localization (Fig. 2, G and H). As control, transfection of a sense (inactive) oligonucleotide for moesin had no effect on cytoskeletal changes or WAVE1 expression during E2 administration (Fig. 2, G and H).

Estrogen regulates WAVE1 via estrogen receptor α (ERα)

Phosphorylation of WAVE1 induced by E2 identified as a shift in the gel migration was prevented by the pure ER antagonist ICI 182,780 (100 nM) (Fig. 3A). To identify which ER isoform mediates the signaling of E2 to WAVE1, we used the preferential ERα agonist 4,49,40-(4 propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT, 10 nM) or the ERβ agonist 2,3-bis(4-hydroxyphenil)-propionitrile (DPN, 10 nM). WAVE1 phosphorylation was detected only in the presence of E2 or PPT, suggesting that ERα supports the signaling to WAVE1, whereas ERβ is not required (Fig. 3A). To substantiate this observation, we silenced ERα in cortical neurons with siRNAs. This resulted in a reduction of ERα expression, along with a dramatic decrease in WAVE1 phosphorylation on all three serine sites during exposure to estrogen (Fig. 3, B and C). This happened in the absence of modifications of the expression of WAVE1 or ERβ (Fig. 3B).

In agreement, remodeling of actin and cell membrane localization of WAVE1 induced by E2 were prevented by ICI 182,780 and supported by the ERα agonist PPT but not by the ERβ-selective agonist DPN (supplemental Fig. B).

ERα signals to WAVE1 via a G protein/c-Src-initiated cascade

To look for the cascades involved in the signaling of ERα to WAVE1 in neurons, we used a variety of approaches to interfere with E2 signaling to WAVE1. PD98059 (5 mM), which inhibits the ERK1/2 MAPK, and wortmannin (30 nM), an inhibitor of phosphatidylinositol-3 kinase, were unable to reduce the phosphorylation of WAVE1 by E2 (supplemental Fig. C).

WAVE1 phosphorylation was instead prevented by the G protein inhibitor pertussis toxin (PTX, 100 ng/ml) and by the inhibitor of c-Src, PP2 (0.2 μM) (supplemental Fig. C), indicating that ERα recruits a G protein/c-Src cascade to WAVE1.

Interaction of ERα with Gα_i and Gβ is required to E2 signaling to c-Src

Consistent with the previous results, E2 administration resulted in c-Src phosphorylation in neurons (Fig. 4A). c-Src phosphorylation was prevented by PTX and by inactivating the G proteins Gα_i

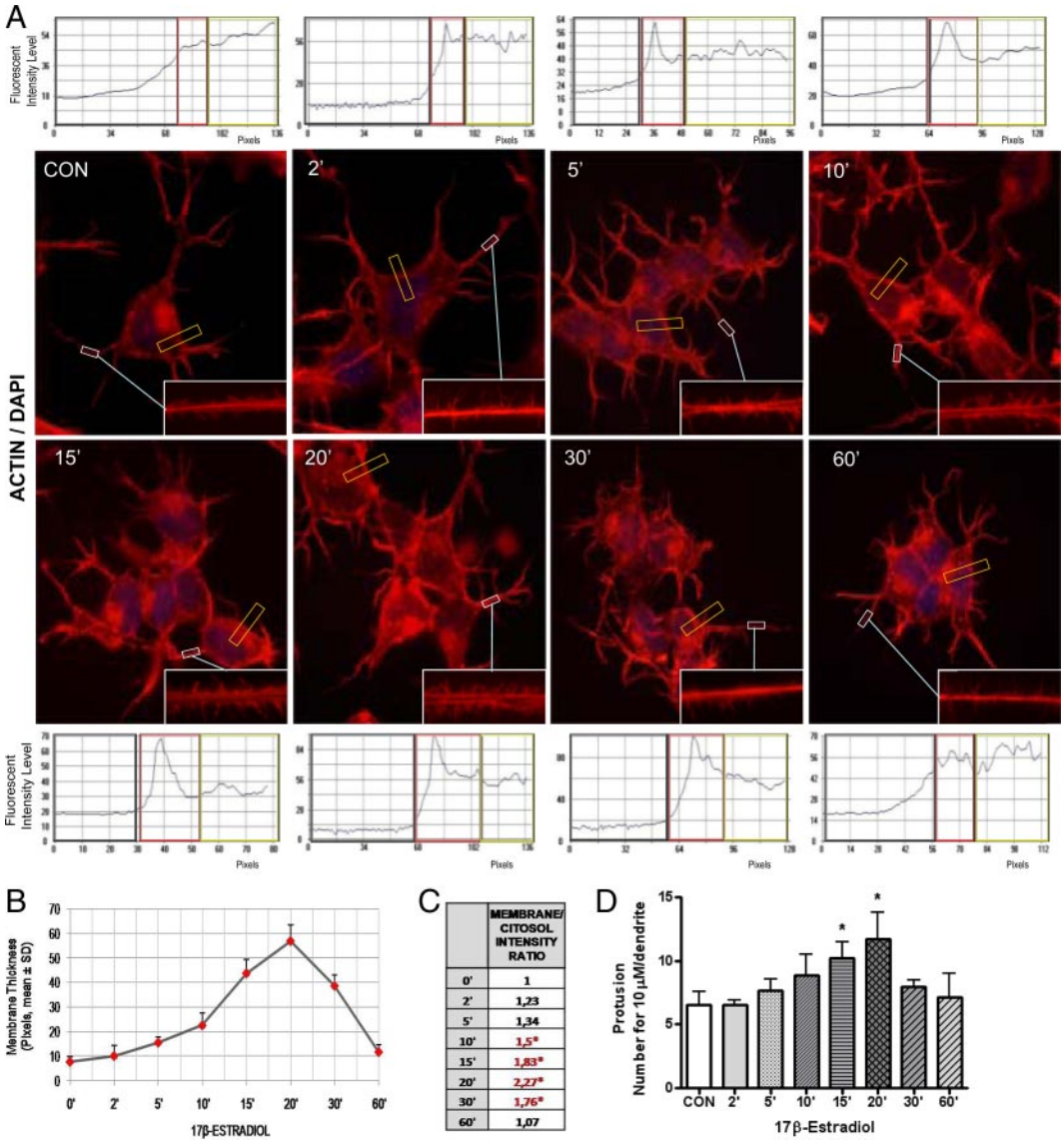

FIG. 1. E2 induces dendritic spine formation and dynamic actin remodeling in cortical neurons. A, Neuronal cells were treated for different times with E2 (10 nM). Actin fibers were stained with phalloidin linked to Texas Red (*red labeling*), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue labeling*). The *insets* show dendritic spines at higher magnification from the *white boxes*. The *yellow boxes* on the cells indicate a sample cellular area that is analyzed in the *colored* graphs. In these graphs, the *longitudinal axis* represents the gray level, and the *horizontal axis* indicates pixels. *Blue*, *red*, and *yellow* areas indicate the extracellular, plasma membrane, and cytoplasmic fractions, respectively. B and C, Mean intensity ratio of actin staining in the membrane/cytoplasm in the same experiment. The results are derived from the sampling of five areas of the cell membrane of 40 different random cells. Areas of minimum and maximum cell membrane thickness were always included. The results are expressed as the mean ± SD of the measurements. *, $P < 0.05$ *vs.* 0 min. D, Mean number (±SD) of dendritic protrusions per 10 μm dendrite length. *, $P < 0.05$ *vs.* 0 min. CON, Control.

and Gβ with either a dominant-negative construct or with specific siRNAs but not by inactivating Gα_{13} with a dominant-negative construct (Fig. 4A). The interaction of ERα with Gα_i and G$\beta\gamma$ has been recently reported in endothelial cells (13). With co-immunoprecipitation experiments, we also find a ligand-induced interaction of ERα with Gα_{i1} and Gβ_1 (Fig. 4, B and C). During E2-induced binding of ERα with Gβ_1, the interaction of Gα_{i1} and Gβ_1 is decreased (Fig. 4C). Consistent with our previous results in endothelial and breast cancer cells (7–9), ERα is also able to interact with Gα_{13} in the presence of E2 (Fig. 4D).

c-Src is a transducer of signals from G proteins to small GTPases (14), and WAVE is involved in actin reorganization and in the formation of membrane ruffles induced by the small GTPase Rac1 (15). We thus studied whether Rac1 is recruited during the signaling of ERα to WAVE1. Indeed, during treatment with E2, cortical neurons transfected with a Rac1 dominant-negative construct did not show an enhanced WAVE1 phosphorylation but actually displayed a slight decrease *vs.* baseline (Fig. 4E). Rac1 is recruited downstream of c-Src, because the Rac1 dominant-negative construct does not impair activation of c-Src by E2 (supplemental Fig. D).

Blockade of G proteins (with PTX), c-Src (with PP2), or Rac1 (with the dominant-negative construct) all resulted in a visible inhibition of WAVE1 membrane translocation and of actin remodeling induced by E2 (supplemental Fig. E), indicating that a signaling cascade involving G proteins, c-Src, and Rac1 mediates the regulatory effects of E2 on WAVE1 in cortical neurons.

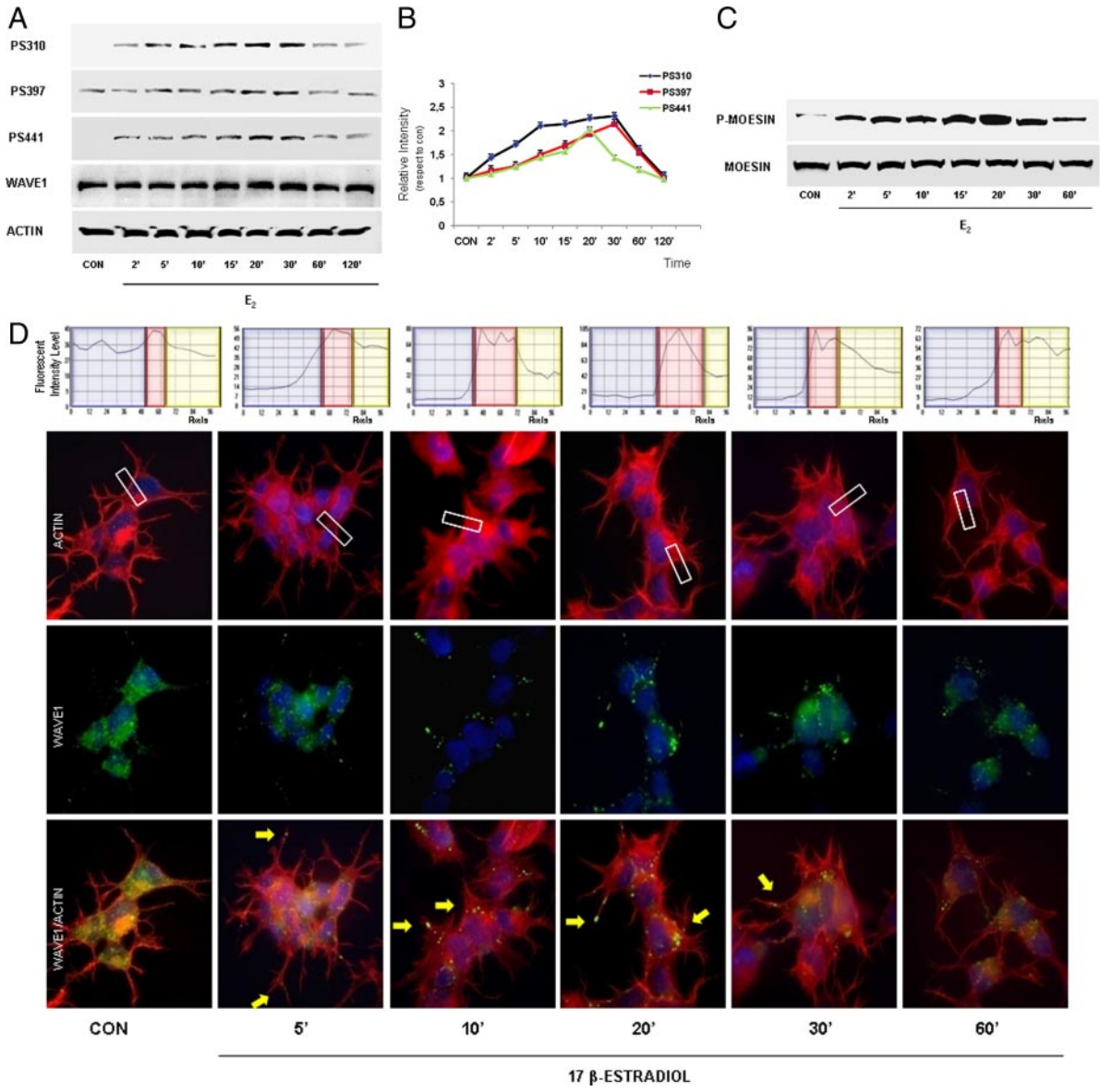

FIG. 2. Estrogen activates actin remodeling in neurons via WAVE1 and moesin. A, Protein extracts from neuronal cells treated with 10 nM E2 for 0–120 min were assayed with Western analysis for their overall content of WAVE1, actin, or P-Ser^{310}-, P-Ser^{397}-, and P-Ser^{441}-WAVE1. B, Densitometric analysis of the P-WAVE1 bands in A. C, Western analysis for wild-type and Thr^{558}-P-moesin of neuronal protein extracts treated with E2 (10 nM) for 0–60 min. D, Neuronal cells were treated for different times with E2 (10 nM). Actin fibers were stained with phalloidin linked to Texas Red (*red labeling*), WAVE1 was stained with fluorescein isothiocyanate (*green labeling*), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue labeling*). E, Mean ± SD thickness of the cell membrane and mean number ± SD of membrane-localized WAVE1, estimated as WAVE1 spots at the cell membrane per cell, indicated as the mean of the measurement of 40 different cells per condition. F, Mean intensity ratio of actin staining in the membrane/cytoplasm in the same experiment. The results are derived from the sampling of five areas of the cell membrane of 40 different random cells. Areas of minimum and maximum cell membrane thickness were always included. *, $P < 0.05$ *vs.* 0 min. G, Neuronal cells were transfected with siRNA *vs.* WAVE1 or with antisense or sense PONs toward moesin for 48 h. WAVE1 expression and cellular localization status was checked by staining for WAVE1 (fluorescein isothiocyanate). Actin fibers were stained with phalloidin-Texas Red, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. H, Mean ± SD thickness of the cell membrane as well as the intensity of actin staining in the cytoplasm and membrane in the same experiment. Technical details are as in E and F. D and G, The *white boxes* on the cells indicate a sample cellular area that is analyzed in the *colored* graphs. In these graphs, the *longitudinal axis* represents the gray level, and the *horizontal axis* indicates pixels. *Blue*, *red*, and *yellow* areas indicate the extracellular, plasma membrane, and cytoplasmic fractions, respectively. CON, Control.

Estrogen signals to WAVE1 via cyclin-dependent kinase 5 (Cdk5)

Cdk5 phosphorylates WAVE1 on the serine residues Ser^{310}, Ser^{397}, and Ser^{441} (12). To test the requirement of Cdk5 for estrogen signaling in neuronal cells, we immunoprecipitated Cdk5 and used the immunoprecipitates (IPs) to perform kinase assays using dephosphorylated histone H1 as the target for serine phosphorylation. E2 administration resulted in a rapid increase in Cdk5 activity, which was sensitive to roscovitine (50 μM), a selective inhibitor of Cdk5 (Fig. 5A). Cdk5 activation in the presence of E2 was also prevented by the c-Src inhibitor (PP2) as well as by transfection of a Rac1 dominant-negative construct (Fig. 5B), overall suggesting that ERα recruitment of c-Src and Rac1 leads to Cdk5 activation.

Consistent with this hypothesis, WAVE1 phosphorylation on the three Cdk5 target sites induced by E2 was inhibited by

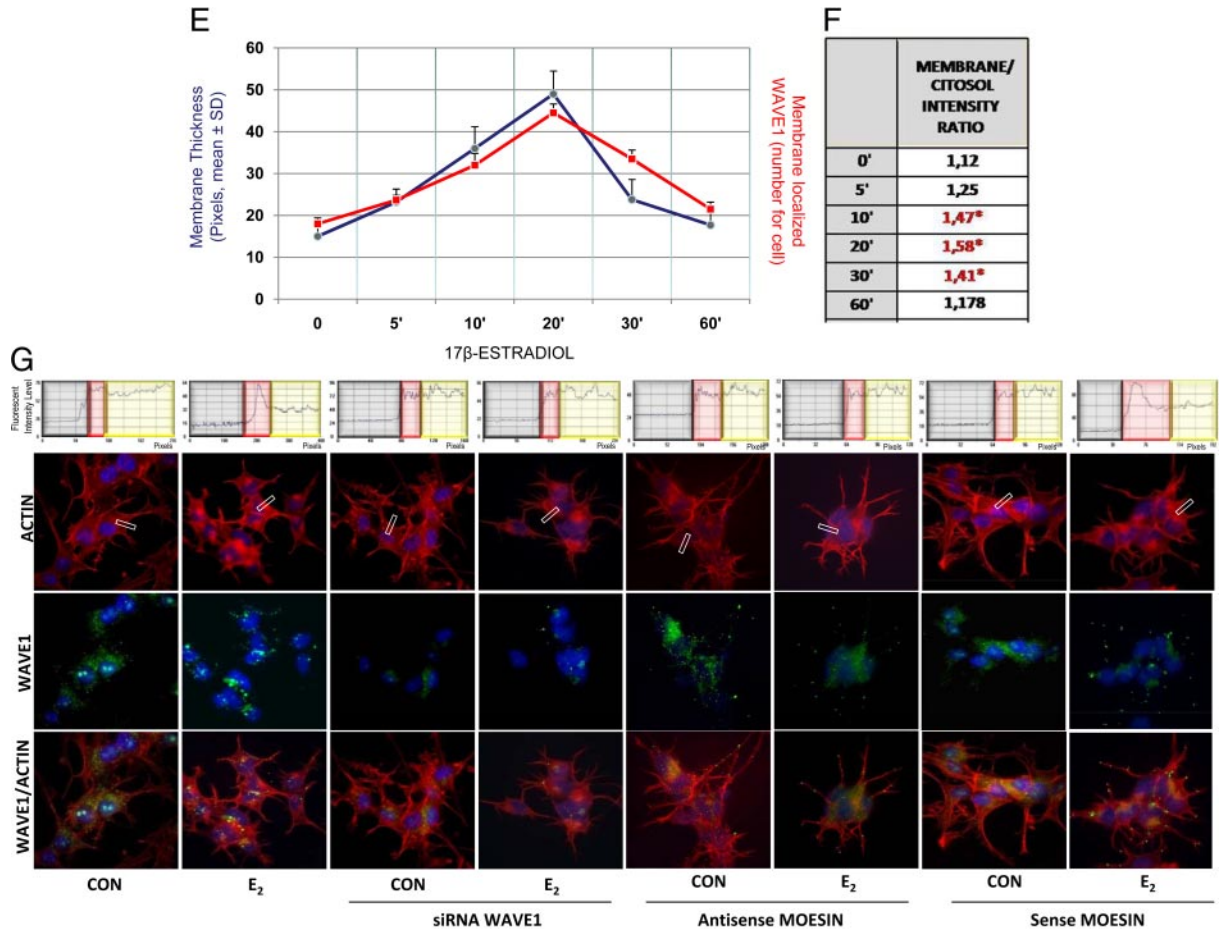

FIG. 2. *Continued*

roscovitine (Fig. 5C). In addition, transfection of neurons with three constructs encoding for WAVE1 with serine to alanine mutations (12) deleting the Cdk5 serine phosphorylation sites (Fig. 5D) resulted in decreased dendritic spine density, particularly with the mutated Ser^{310} construct (S310A-WAVE1) (Fig. 5, E and F), confirming the hypothesis that E2 regulates WAVE1 and dendritic spine formation via Cdk5.

Differential signaling of ERα to moesin and WAVE1 in neurons

To confirm that ERα recruits different signaling avenues to regulate moesin and WAVE1, we exposed neurons to E2 (10 nM) during the blockade of $G\alpha_i$, $G\beta$, c-Src, Rac1, and Cdk5 (involved in the signaling to WAVE1) or of $G\alpha_{13}$, RhoA, or Rho-associated kinase (involved in signaling to moesin). Western analysis of WAVE1 and moesin phosphorylation showed that the blockade at any level of the c-Src/Rac1/Cdk5 cascade results in impaired phosphorylation of WAVE1 but not of moesin (Fig. 5G). Conversely, blockade of the $G\alpha_{13}$/RhoA/Rho-associated kinase impairs moesin phosphorylation by E2 but not WAVE1 phosphorylation (Fig. 5G). Blockade of $G\alpha_i$ or $G\beta$ resulted in inhibition of both moesin and WAVE1 phosphorylation, suggesting that these two G proteins may be involved in downstream signaling to these actin regulators via multiple pathways.

Estrogen signaling to WAVE-1 results in Arp-2/3 membrane translocation

Actin nucleation by the Arp-2/3 complex is critical for the rapid formation of an actin network at the leading edge of cells (16). In response to Rac1, the Arp-2/3 complex initiates the formation of lamellipodia. WAVE1 acts as a scaffolding complex relaying signals from small GTPases to the Arp-2/3 complex (17).

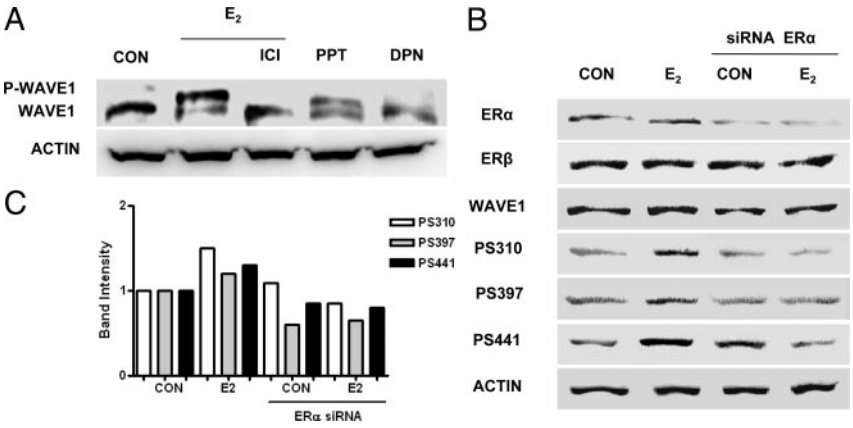

FIG. 3. Estrogen signals to WAVE1 via ERα. A, Cortical neurons were exposed for 20 min to 10 nM E2 in the presence or absence of the pure ER antagonist ICI 182,780 (ICI; 100 nM) or with the ERα-selective ligand PPT (10 nM) or the ERβ-selective agonist DPN (10 nM). Total phosphorylation of WAVE1 was assayed with Western analysis as a gel migration shift in the band. B, Cortical neurons were transfected with siRNA *vs.* ERα (siRNA ERα) or with vehicle, and protein analysis for ERα, ERβ, actin, or wild-type (WAVE1) or P-Ser^{310}-, P-Ser^{397}-, and P-Ser^{441}-WAVE1 was performed on cell lysates after treatment for 20 min with 10 nM E2. C, The graph displays the quantitative analysis of the intensity of the bands in B, obtained as number of photons measured by the ChemiDoc digital imaging system and evaluated with the Quantity One Software (Bio-Rad, Hercules, CA). CON, Control.

Cortical neurons treated with E2 displayed a membrane translocation of Arp-2 that was time consistent with the phosphorylation of WAVE1 (Fig. 6, A and B). Blockade of c-Src with PP2 or of Rac1 by transfection of a dominant-negative construct or of Cdk5 with roscovitine blocked the estrogen-induced membrane translocation of Arp-2, supporting the concept that estrogen signals to WAVE1 and Arp-2 through a c-Src/Rac1/Cdk5 cascade.

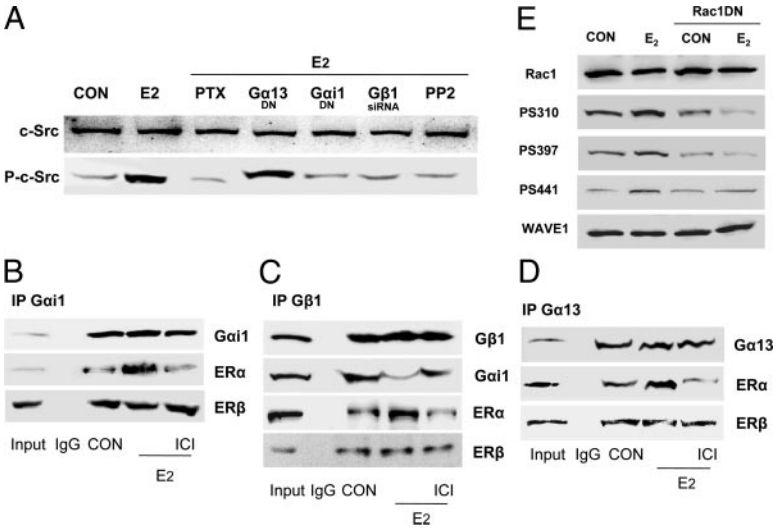

FIG. 4. ERα signals to WAVE1 via Gαi, Gβ, c-Src, and Rac1. A, Cortical neuronal cells were exposed for 20 min to 10 nM E2, in the presence or absence of the G protein inhibitor PTX (100 ng/ml) or the c-Src inhibitor PP2 (0.2 μM) or after transfection with dominant-negative Gα13 or Gαi constructs or siRNAs *vs.* Gβ1. Wild-type c-Src or P-Tyr^{416}-c-Src were assayed in cell extracts. B–D, Neuronal cell protein extracts were immunoprecipitated with antibodies toward the indicated G proteins and co-immunoprecipitation of ERα, ERβ, and Gαi (C) was tested by Western analysis. E, Cortical neurons were treated for 20 min with 10 nM E2, with or without transfection of a dominant-negative Rac1 for 48 h. Rac1, wild-type WAVE1, or P-Ser^{310}-, P-Ser^{397}-, and P-Ser^{441}-WAVE1 were assayed in cell extracts. CON, Control.

Estrogen regulates the formation of dendritic spines via WAVE1 and moesin in cortical neurons

The formation of excitatory synaptic connections in neurite extensions is dependent on the development of actin-rich dendritic spines (18). Actin staining in cortical neurons demonstrated a net enrichment in dendritic spine density after treatment with E2 (Fig. 7, A and B). Silencing of WAVE1 with siRNAs significantly decreased the number of spines induced by E2 (Fig. 7, A and B). As control, the transfection of a siRNA-resistant WAVE1 construct reversed the effect of the siRNA (Fig. 7, A and B). Moesin silencing with specific PONs also prevented the increase in dendritic spines by E2 (Fig. 7, A and B).

Discussion

The control of cell morphology and of the interaction of the cell with the extracellular environment requires a number of regulators orchestrating the different cytoskeletal components and their interactions with the cell membrane and with anchorage proteins (6). These phenomena are key determinants of brain plasticity, making cognition, memory, or learning possible via neuronal/glial remodeling (5). Dendrites are the sites where neurons receive, process, and integrate inputs from their multiple presynaptic partners. Both the shape of the dendritic trees and the density and shape of their spines can undergo significant changes during the development and life of a neuron. Dendritic spines are composed mainly of actin microfilaments that undergo a continuous remodeling that is important for the development of neuronal circuits (19).

Estrogens stimulate the formation of dendritic spines as early as during embryonic neuronal development and continue to act on mature neurons regulating their structural plasticity at mature synapses and increasing dendritic spine density through the dynamic control of actin filaments (20).

The key finding of this work is that ERα uses the actin controllers WAVE1 and moesin in neurons to induce a rapid cytoskeletal remodeling that supports the formation of dendritic spines. In the presence of its ligand, ERα recruits a Gα$_i$-Gβ/c-Src/Rac1/Cdk5/WAVE1 cascade (Fig. 8). This turns into WAVE1 phosphorylation and translocation to the sites where the actin cytoskeleton and the cell membrane are actively remodeled and in the parallel membrane localization of the Arp-2/3 protein complex, which is responsible for the extension and branching of actin filaments and thus for cell membrane remodeling. The parallel recruitment of the Ezrin/Radixin/Moesin (ERM) actin-binding pro-

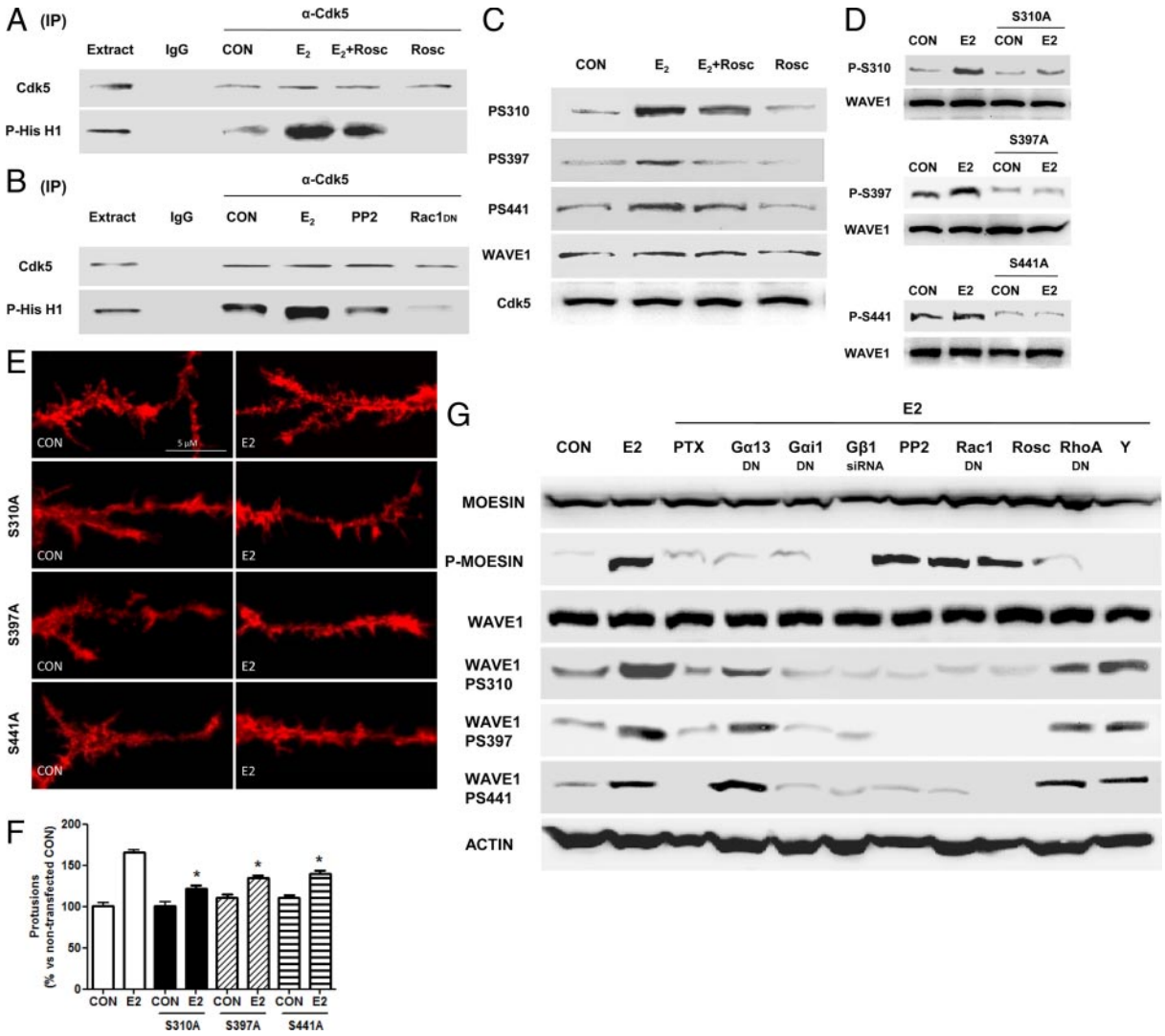

FIG. 5. Estrogen signals to WAVE1 via a Gαi-Gβ/c-Src/Rac1/Cdk5 cascade, whereas signaling to moesin requires a Gα13/RhoA/ROCK2 pathway. A–C, Cortical neurons were treated with E2 in the presence or absence of the specific Cdk5 inhibitor roscovitine (Rosc; 50 μM) or the c-Src inhibitor PP2 (0.2 μM) or after transfection with a dominant-negative Rac1 construct. A and B, Whole protein extracts were immunoprecipitated with an antibody against Cdk5. The IPs were used in a kinase assay to phosphorylate histone H1 (substrate of Cdk5). Phosphorylated histone H1 was detected after Western analysis with a phosphospecific antibody. C, Neuronal cell content of Cdk5, WAVE1, and P-Ser^{310}-, P-Ser^{397}-, and P-Ser^{441}-WAVE1 were assayed with Western analysis. D–F, Cortical neurons were exposed to 10 nM E2 for 20 min with or without transfection of WAVE1 constructs with inactivating mutations of the Ser^{310}, Ser^{397}, and Ser^{441} Cdk5 phosphorylation sites. D, Western analysis of wild-type or P-Ser^{310}-, P-Ser^{397}-, or P-Ser^{441}-WAVE1 was performed. E, Dendritic spine morphology and number were measured with immunofluorescence after staining actin fibers with phalloidin/Texas Red. *Scale bar*, 5 μm. F, The graph shows the quantitative analysis of spine density calculated as the number of spines per 10 μm dendrite length, normalized *vs.* nontransfected, vehicle-treated neurons and expressed as a percentage. The results are expressed as the mean ± SD. *, $P < 0.05$ *vs.* E2 in nontransfected cells. G, Cortical neurons were treated with E2 in the presence or absence of the G protein inhibitor PTX (100 ng/ml), the c-Src inhibitor PP2 (0.2 μM), the Cdk5 inhibitor roscovitine (Rosc; 50 μM), or the Rho-associated kinase inhibitor (ROCK-2) Y-27632 (Y, 10 μM) or after transfection with dominant-negative Gα13, Gαi, Rac1, or RhoA constructs or with siRNAs *vs.* Gβ1. Western analysis for actin, wild-type, and Thr^{558}-P-moesin, wild-type, and P-Ser^{310}-, or P-Ser^{397}-, or P-Ser^{441}-WAVE1 was performed. CON, Control.

tein, moesin, via a $G\alpha_{13}$/RhoA/ROCK-2 cascade (Fig. 8) contributes to the remodeling of the cytoskeleton and is required for the estrogen-induced dendritic spine formation.

Recent evidence on cultured neurons and in intact mice shows that loss of WAVE1 results in a dramatic decrease in mature dendritic spines (12), suggesting that in neurons, the regulation of the actin cytoskeleton is critical for the development and maintenance of these structures. Thus, the finding that estrogen controls neuronal cell morphology through the rapid modulation of WAVE1 heralds promises from both the biological and medical perspective. Indeed, WAVE1 knockout mice exhibit deficits in learning and memory (21), suggesting the intriguing hypothesis that the increased prevalence of cognitive impairment and of some degenerative disorders observed in conditions of estrogen withdrawal might to some extent be due to lack of WAVE1 control by these steroids in brain cells.

When E2 hits ERα, a signaling cascade to Cdk5 is recruited. This leads to intracellular localization of WAVE1 at the cell membrane where it drives the accumulation of actin filaments and increases the mechanical force necessary for neurite extension and growth-cone translocation. Although all the three identified WAVE proteins are known to localize at growth cones, their respective roles are unclear (22). The WAVE1 complex seems to be transported by kinesin-1, which has been suggested to be particu-

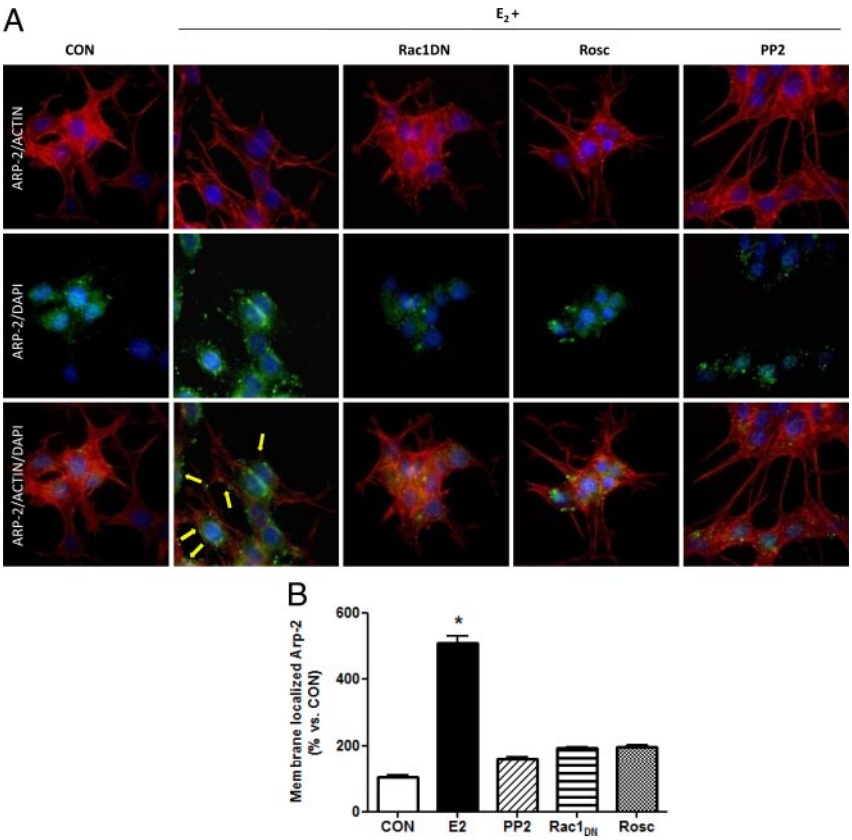

FIG. 6. Estrogen signaling to WAVE1 turns into membrane localization of the Arp-2/3 complex. Cortical neurons were treated for 20 min with 10 nM E2 in the presence or absence of the Cdk5 inhibitor roscovitine (Rosc; 20 μM) or the c-Src inhibitor PP2 (0.2 μM) or after transfection with a dominant-negative Rac1 construct. A, Cells were stained with an antibody against Arp-2 (fluorescein isothiocyanate), actin fibers were stained with phalloidin linked to Texas Red, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. *Yellow arrows* indicate membrane-localized Arp-2. B, Quantification of the membrane-localized Arp-2 complexes in the different conditions. Results are expressed as percent *vs.* control cells (mean ± SD). *, $P < 0.05$ *vs.* control. Membrane-localized Arp-2 complexes were counted in 40 different cells. CON, Control.

larly important for axon elongation (18), whereas other reports indicate an involvement of neural WASP in spine formation (23). Phosphorylation and dephosphorylation of WAVE1 seem to be necessary for spine formation, as well. During spine formation, the WAVE1 complex is hyperphosphorylated on the proline-rich region by Cdk5, resulting in a later functional regulation of the Arp-2/3 complex (12). Thus, Cdk5-dependent phosphorylation of WAVE1 and the subsequent Arp-2/3-mediated branching of actin is a newly identified mechanism by which dendritic spine formation is regulated by estrogen. It is likely that this phenomenon results from the synergic regulation of multiple converging pathways, as suggested by the seemingly paradoxical reduction of WAVE1 phosphorylation induced by E2 during Rac1 blockade. This suggests that E2 also recruits a yet unidentified inhibitory pathway that reduces Cdk5 phosphorylation of WAVE1 that is independent of Rac1 (Fig. 8). In addition, the requirement of moesin for optimal cytoskeletal remodeling and spine formation also stands for the existence of an integrated signaling network enacted by ERs in neurons to control the architecture of the cytoskeleton.

Increasing evidence indicates that a number of dynamic functional modifications induced by estrogens in neurons are made via rapid extranuclear signaling pathways recruited by ERs at the cell membrane or within the cytoplasm (24, 25), in various cell types, including neurons (26, 27). These rapid signaling actions of ERs render estrogens flexible modulators of neuronal function. It is intriguing to find that the modifications of the neuronal cytoskeleton and cell membrane are achieved through this category of action of ERs. Whether such rapid modifications of the structure of neuronal membrane and of the number of dendritic spines can in any way be related to changes in neuronal function that could lead to improved or preserved cognition or memory is difficult to say. However, it is well established that brain function undergoes rapid modifications in the presence of changing estrogen concentrations, as shown by animal models and by the brain-originated disturbances (*e.g.* vasomotor symptoms) seen after delivery or menopause, supporting the idea that sex steroids might have a role as fast regulators of central nervous system function.

In conclusion, we here show that estrogens are implicated in neurite extension in neuronal cells acting through rapid, extranuclear signal transduction pathways recruited by ERα. Estradiol acts through the activation of G proteins via ERα, leading to the recruitment of a c-Src/Rac1/Cdk5/WAVE1/Arp-2/3 cascade. Through this cascade, E2 supports the rearrangement of the actin cytoskeleton at sites of ongoing spine formation in cortical rat neurons. The parallel G protein-dependent activation of the RhoA/ROCK-2/moesin cascade contributes to this phenomenon. The identification of these original rapid actions of estrogen in brain cells increases our understanding of the complex actions of sex steroids in the central nervous system and may provide new tools to interfere with some of the gender-related degenerative changes observed in the brain throughout aging.

Materials and Methods

Neuronal cell cultures

Primary cultures of cortical neurons were obtained from embryonic d-18 rat fetuses as described (28).

Immunoblotting

Immunoblotting was performed with antibodies against histone H1, phospho-histone H1 (P-Ser^{27}), and WAVE1 (Sigma-Aldrich Laboratories, St. Louis, MO); actin (C-11), Arp-2 (H-84), ERα (H-184), ERβ (N-19), c-Src (H-12), Rac1 (C-14), Gα_{i1} (R4), Gβ_1 (C-16), and Gα_{13} (A-20) (Santa Cruz Biotechnology, Santa Cruz, CA); Cdk5 (268-283) (Calbiochem, La Jolla, CA); phospho-Src (Tyr^{416}) (Cell Signaling Technology, Beverly, MA); WAVE1 (c-term), phospho-Ser^{310}-WAVE1, phospho-Ser^{397}-WAVE1, phospho-Ser^{441}-WAVE1, and moesin (clone 38; Transduction Laboratories, Lexington, KY); and Thr^{558}-P-moesin

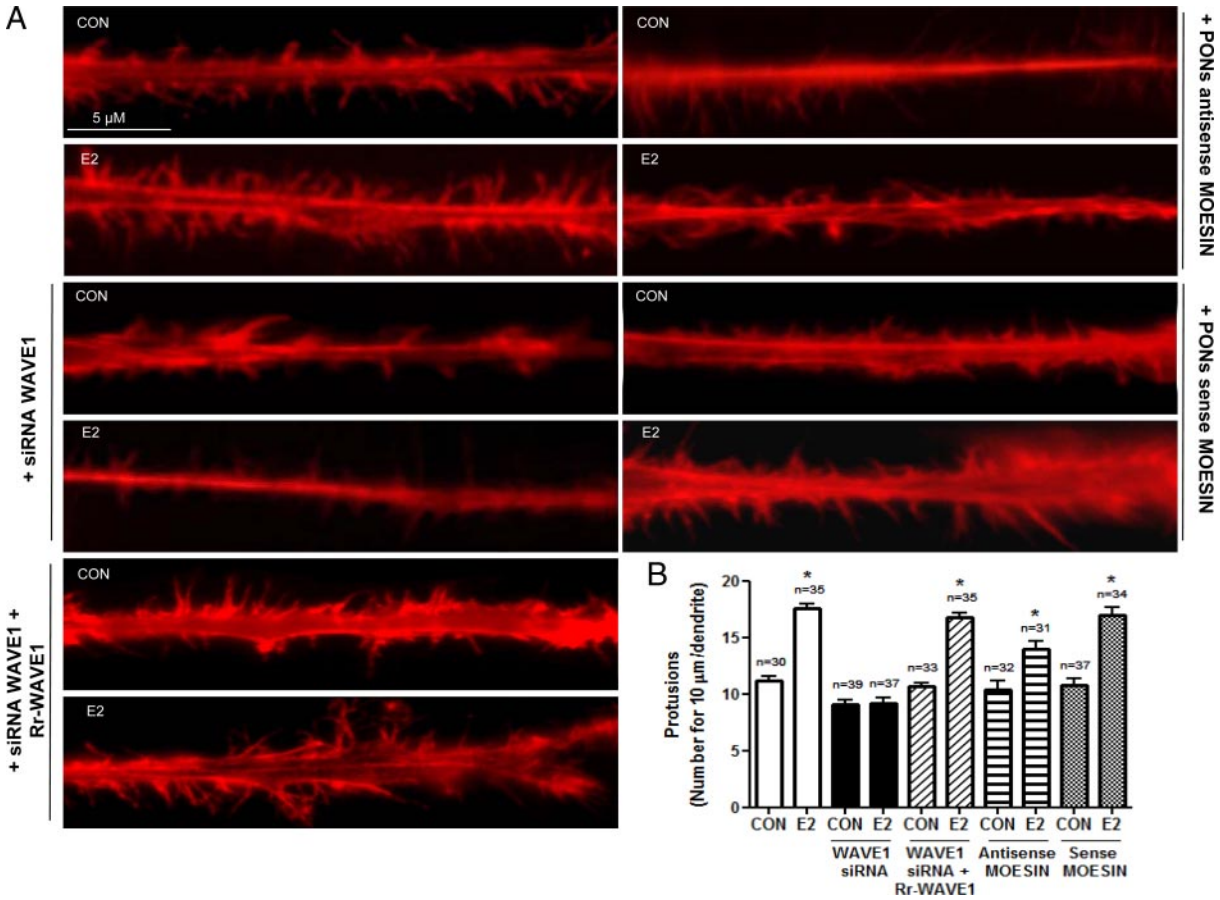

FIG. 7. Estrogen increases dendritic spine density via WAVE1 and moesin. Cortical neurons were incubated in the presence of 10 nM E2 for 20 min in baseline conditions or after silencing of WAVE1 with specific siRNAs or of moesin with antisense PONs Sense PONs for moesin served as control. A, Dendritic spine morphology and number were measured with immunofluorescence after staining actin fibers with phalloidin/Texas Red. *Scale bar*, 5 μm. B, The graph shows the quantitative analysis of spine density expressed as the number of spines per 10 μm dendrite length. The results are expressed as the mean ± SD. *, $P < 0.05$ *vs.* control. CON, Control.

(sc-12895; Santa Cruz). Each blot was performed individually, without stripping and reprobing membranes.

Cell immunofluorescence

Fixed cells on coverslips were incubated with anti-WAVE1 (1:750; Sigma-Aldrich) and anti-Arp-2 (H-84) (1:300; Santa Cruz). Immunofluorescence was visualized using an Olympus BX41 microscope.

Quantitative analysis of cell membrane morphology and thickness and of actin fiber remodeling

The remodeling of actin fibers and the morphological changes of the membrane were quantified by assessing the intensity of actin fluorescence using the Leica QWin software (Leica Microsystems, Wetzlar, Germany). This analysis was performed by randomly measuring fluorescence using boxes of a fixed size including the extra- and intracellular space across the membrane, and by recording the intensity of the signal in a spatial-related fashion. In other words, the program provides a graph where the pixel fluorescence intensity is linearly recorded. By visually selecting the inner and outer parts of the membrane in each microphotograph, we were able to quantify the thickness of the membrane as sampled in each box. In addition, the measure of the mean fluorescent intensity in the areas identified as membrane space or intracellular space within the box was used to quantify the amount of cytosolic *vs.* membrane-associated actin. Using these boxes, we sampled five areas per cell, and we repeated this on 40 different cells per experimental condition. Number of spine protrusions per 10 μm dendrite length were measured in five different dendrites per cell and repeated in 40 different cells.

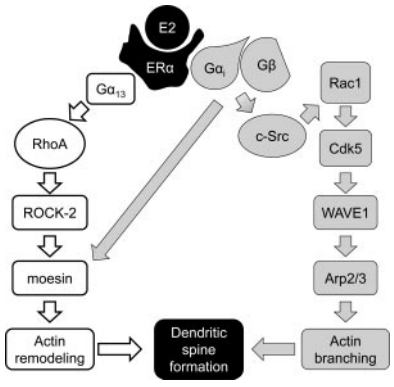

FIG. 8. Signaling cascades of ERα to WAVE1 and moesin and dendritic spine formation in cortical neurons.

Immunoprecipitation

Primary cortical neurons were harvested with IP lysis buffer. The immunoprecipitating antibodies were added in 500 μl of lysis buffer for 1 h at 4 C with gentle rocking, and 40 μl 1:1 protein A-agarose was added and rocked for 2 additional hours. The mixture was centrifuged, and the IPs were washed. Immunoprecipitated proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with antibodies toward the co-immunoprecipitating proteins, and Western analysis was performed.

Kinase assays

Neurons were harvested, equal amounts of lysates were immunoprecipitated with anti-Cdk5 (2 μg) antibody, 40 μl 1:1 protein A-agarose was added, and the IPs were washed three times. Two additional washes

were performed, and the samples were resuspended with 5 μg dephosphorylated histone H1 (H4524; Sigma-Aldrich) together with 500 μM ATP and 75 mM $MgCl_2$. The reaction was stopped on ice and by resuspending the samples in Laemmli buffer. Western analysis was performed for Cdk5 (268-283; Calbiochem) and anti-phospho-histone H1.4 ($pSer^{27}$) (H7664; Sigma-Aldrich).

Gene silencing and protein inactivation

Synthetic siRNAs targeting ERα (siRNA SMARTpool ESR1; Dharmacon, Lafayette, CO), WAVE1 (siRNA SMARTpool WAVE1; Dharmacon), or Gβ1 (sc-41762; Santa Cruz) were used at the final concentration of 50–75 nM. Two synthetic siRNAs targeting ERα (siRNA SMARTpool ESR1; Dharmacon) were used at the final concentration of 100 nM to silence ERα. Validated antisense PONs (S-modified) complementary to the 1-15 position of the human moesin gene coding region were obtained. The sequence was 5′-TACGGGTTTTGCTAG-3′ for moesin antisense PON. The complementary sense PON was used as control (5′-CTAGCAAAAC-CCGTA-3′). Cortical neurons were treated 48 h after siRNA or PON transfection. The efficacy of gene silencing was checked with Western analysis and found to be optimal at 48 h.

Transfection experiments

The dominant-negative constructs for $G\alpha_{i1}$ ($G\alpha_{i1}$ G202T), Rac1 (Rac1 T17N), RhoA (RhoA T19N), and $G\alpha_{13}$ ($G\alpha_{13}$ Q226L/D294N) were from the Guthrie cDNA Resource Center (www.cdna.org). The inserts were cloned in pcDNA3.1+. The plasmids (10 μg) were transfected into cortical neurons using Lipofectamine (Invitrogen, Carlsbad, CA). Parallel cells were transfected with empty pcDNA3.1+ plasmid. Cells (60–70% confluent) were treated 24 h after transfection.

Statistical analysis

All values are expressed as mean ± SD. Statistical analyses and graphics were done using InStat from GraphPad Prism Software. Statistical differences between mean values were determined by ANOVA, followed by the Fisher's protected least significance difference (PLSD).

For additional detail, see supplemental Materials and Methods.

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Address all correspondence and requests for reprints to: Tommaso Simoncini, M.D., Ph.D., Molecular and Cellular Gynecological Endocrinology Laboratory (MCGEL), Department of Reproductive Medicine and Child Development, Division of Obstetrics and Gynecology, University of Pisa, Via Roma 57, 56100 Pisa, Italy. E-mail: t.simoncini@obgyn.med.unipi.it.

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References

1. **McEwen B** 2002 Estrogen actions throughout the brain. Recent Prog Horm Res 57:357–384
2. **Hojo Y, Murakami G, Mukai H, Higo S, Hatanaka Y, Ogiue-Ikeda M, Ishii H, Kimoto T, Kawato S** 2008 Estrogen synthesis in the brain: role in synaptic plasticity and memory. Mol Cell Endocrinol 290:31–43
3. **Sherwin BB, Henry JF** 2008 Brain aging modulates the neuroprotective effects of estrogen on selective aspects of cognition in women: a critical review. Front Neuroendocrinol 29:88–113
4. **Vegeto E, Benedusi V, Maggi A** 2008 Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. Front Neuroendocrinol 29:507–519
5. **Duffau H** 2006 Brain plasticity: from pathophysiological mechanisms to therapeutic applications. J Clin Neurosci 13:885–897
6. **Pollard TD, Borisy GG** 2003 Cellular motility driven by assembly and disassembly of actin filaments. Cell 112:453–465
7. **Simoncini T, Scorticati C, Mannella P, Fadiel A, Giretti MS, Fu XD, Baldacci C, Garibaldi S, Caruso A, Fornari L, Naftolin F, Genazzani AR** 2006 Estrogen receptor α interacts with Gα13 to drive actin remodeling and endothelial cell migration via the RhoA/Rho kinase/moesin pathway. Mol Endocrinol 20:1756–1771
8. **Giretti MS, Fu XD, De Rosa G, Sarotto I, Baldacci C, Garibaldi S, Mannella P, Biglia N, Sismondi P, Genazzani AR, Simoncini T** 2008 Extra-nuclear signalling of estrogen receptor to breast cancer cytoskeletal remodelling, migration and invasion. PLoS ONE 3:e2238
9. **Fu XD, Giretti MS, Baldacci C, Garibaldi S, Flamini M, Sanchez AM, Gadducci A, Genazzani AR, Simoncini T** 2008 Extra-nuclear signaling of progesterone receptor to breast cancer cell movement and invasion through the actin cytoskeleton. PLoS ONE 3:e2790
10. **Zsarnovszky A, Horvath TL, Garcia-Segura LM, Horvath B, Naftolin F** 2001 Oestrogen-induced changes in the synaptology of the monkey (*Cercopithecus aethiops*) arcuate nucleus during gonadotropin feedback. J Neuroendocrinol 13:22–28
11. **Suetsugu S, Hattori M, Miki H, Tezuka T, Yamamoto T, Mikoshiba K, Takenawa T** 2002 Sustained activation of N-WASP through phosphorylation is essential for neurite extension. Dev Cell 3:645–658
12. **Kim Y, Sung JY, Ceglia I, Lee KW, Ahn JH, Halford JM, Kim AM, Kwak SP, Park JB, Ho Ryu S, Schenck A, Bardoni B, Scott JD, Nairn AC, Greengard P** 2006 Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology. Nature 442:814–817
13. **Kumar P, Wu Q, Chambliss KL, Yuhanna IS, Mumby SM, Mineo C, Tall GG, Shaul PW** 2007 Direct interactions with Gαi and G$\beta\gamma$ mediate nongenomic signaling by estrogen receptor α. Mol Endocrinol 21:1370–1380
14. **Jaffe AB, Hall A** 2005 Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 21:247–269
15. **Stradal TE, Rottner K, Disanza A, Confalonieri S, Innocenti M, Scita G** 2004 Regulation of actin dynamics by WASP and WAVE family proteins. Trends Cell Biol 14:303–311
16. **Higgs HN, Pollard TD** 1999 Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. J Biol Chem 274:32531–32534
17. **Takenawa T, Miki H** 2001 WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. J Cell Sci 114:1801–1809
18. **Soderling SH, Guire ES, Kaech S, White J, Zhang F, Schutz K, Langeberg LK, Banker G, Raber J, Scott JD** 2007 A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory. J Neurosci 27:355–365
19. **Matus A** 2000 Actin-based plasticity in dendritic spines. Science 290:754–758
20. **Woolley CS, McEwen BS** 1993 Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol 336:293–306
21. **Soderling SH, Langeberg LK, Soderling JA, Davee SM, Simerly R, Raber J, Scott JD** 2003 Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice. Proc Natl Acad Sci USA 100:1723–1728
22. **Nozumi M, Nakagawa H, Miki H, Takenawa T, Miyamoto S** 2003 Differential localization of WAVE isoforms in filopodia and lamellipodia of the neuronal growth cone. J Cell Sci 116:239–246
23. **Udo H, Jin I, Kim JH, Li HL, Youn T, Hawkins RD, Kandel ER, Bailey CH** 2005 Serotonin-induced regulation of the actin network for learning-related synaptic growth requires Cdc42, N-WASP, and PAK in Aplysia sensory neurons. Neuron 45:887–901
24. **Fu XD, Simoncini T** 2008 Extra-nuclear signaling of estrogen receptors. IUBMB Life 60:502–510
25. **Levin ER** 2008 Rapid signaling by steroid receptors. Am J Physiol Regul Integr Comp Physiol 295:R1425–R1430
26. **Zhao L, Brinton RD** 2007 Estrogen receptor α and β differentially regulate intracellular Ca^{2+} dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. Brain Res 1172:48–59
27. **Mannella P, Sanchez AM, Giretti MS, Genazzani AR, Simoncini T** 2009 Oestrogen and progestins differently prevent glutamate toxicity in cortical neurons depending on prior hormonal exposure via the induction of neural nitric oxide synthase. Steroids 74:650–656
28. **Brewer GJ** 1997 Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71:143–155