Estrogen Levels Regulate the Subcellular Distribution of Phosphorylated Akt in Hippocampal CA1 Dendrites

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In addition to genomic pathways, estrogens may regulate gene expression by activating specific signal transduction pathways, such as that involving phosphatidylinositol 3-kinase (PI3-K) and the subsequent phosphorylation of Akt (protein kinase B). The Akt pathway regulates various cellular events, including the initiation of protein synthesis. Our previous studies showed that synaptogenesis in hippocampal CA1 pyramidal cell dendritic spines is highest when brain estrogen levels are highest. To address the role of Akt in this process, the subcellular distribution of phosphorylated Akt immunoreactivity (pAkt-I) in the hippocampus of female rats across the estrous cycle and male rats was analyzed by light microscopy (LM) and electron microscopy (EM). By LM, the density of pAkt-I in stratum radiatum of CA1 was significantly higher in proestrus rats (or in estrogen-supplemented ovariectomized females) compared with diestrus, estrus, or male rats. By EM, pAkt-I was found throughout the shafts and in select spines of stratum radiatum dendrites. Quantitative ultrastructural analysis identifying pAkt-I with immunogold particles revealed that proestrus rats compared with diestrus, estrus, and male rats contained significantly higher pAkt-I associated with (1) dendritic spines (both cytoplasm and plasmalemma), (2) spine apparati located within 0.1 μm of dendritic spine bases, (3) endoplasmic reticula and polyribosomes in the cytoplasm of dendritic shafts, and (4) the plasmalemma of dendritic shafts. These findings suggest that estrogens may regulate spine formation in CA1 pyramidal neurons via Akt-mediated signaling events.

Key words: **sex steroids; hippocampus; signal transduction; rat; electron microscopy; protein synthesis**

Introduction

Estrogen-regulated gene expression is one likely mechanism for regulation of synapse formation in the hippocampal formation (McEwen et al., 2001). However, estrogens produce rapid effects at membranes via signal transduction intermediates, including those coupled with G-protein receptors (Kelly and Wagner, 1999; Kelly et al., 1999), and they also regulate gene expression through intracellular signaling cascades (Kelly and Levin, 2001; Lee and McEwen, 2001). Estrogens increase intracellular Ca^{2+} concentrations and activate adenylate cyclase, leading to stimulation of protein kinase A (PKA) and protein kinase C (PKC) pathways (Levin, 2001). Estrogens activate the mitogen-activated protein kinase (MAPK) family and can stimulate the phosphatidylinositol 3-Kinase (PI3-K) pathway leading to activation of the Akt pathway (Toran-Allerand et al., 1999; Honda et al., 2000; Kelly and Levin, 2001; Belcher and Zsarnovszky, 2001). Importantly, estrogen receptor (ER) α, but not ERβ, activates PI3-K through interaction with p85α, a regulatory subunit of PI3-K (Simoncini et al., 2000).

Akt (protein kinase B) is a serine/threonine kinase that mediates the downstream effects of PI3-K, including cell survival and proliferation (Kennedy et al., 1997, 1999), stimulation of glucose transporter 4 translocation (Kohn et al., 1996), inhibition of glycogen synthase kinase-3 (Cross et al., 1995), activation and regulation of endothelial nitric oxide synthase (Dimmeler et al., 1999; Fulton et al., 1999), and hormone-independent activation of ERs (Campbell et al., 2001). Products of PI3-K induce translocation of Akt to the plasma membrane where it is further phosphorylated by two upstream kinases, 3-phosphoinositide-dependent kinases 1 and 2, at Thr-308 and Ser-473, respectively (Downward, 1998, 2001; Gingras et al., 1998; Pugazhenthi et al., 2000). Phosphorylated Akt (pAkt) functions as a regulator of several downstream targets, both nuclear and cytoplasmic. For example, pAkt regulates the inactivation by hyperphosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), an initiation factor-binding protein that acts as a translational repressor in its unphosphorylated state (Gingras et al., 1998).

Previous studies showed a cyclic buildup and breakdown of synapses on CA1 pyramidal cell dendritic spines across the rat estrous cycle, with peak synaptogenesis occurring when brain levels of estrogen are highest (Gould et al., 1990; Woolley and McEwen, 1992; Woolley, 1998). Although our recent ultrastructural studies confirmed the presence of nuclear ERα in inhibitory interneurons, they revealed ERα immunoreactivity at nonnuclear sites in hippocampal CA1 neurons, especially in dendritic spines (Milner et al., 2001). Because estrogens activate Akt via ERα, they may have a role in nongenomic regulation of spine formation (McEwen et al., 2001).

This study addresses whether estrogens activate Akt in hippocampal CA1 neurons and considers the implications of this for the regulation of signaling events by estrogen. First, light microscopic densitometry was used to measure the intensity of pAkt

Received Aug. 5, 2002; revised Dec. 13, 2002; accepted Jan. 2, 2003.

This work was supported by National Institutes of Health Grants NS07080, DA08259 (T.A.M.), HL18974 (T.A.M.), and MH12977 (K.T.A.) and the Ares-Serono Foundation (K.T.A.). We thank Dr. Joseph P. Pierce for his assistance with quantitative immunocytochemical methodology and statistical analysis and Dr. Carrie T. Drake for her helpful suggestions on this manuscript.

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immunoreactivity (pAkt-I) in stratum radiatum of CA1 across the estrous cycle and in male rats. Second, the subcellular distribution of pAkt within dendrites, and its possible redistribution in the presence of estrogens, was determined using quantitative electron microscopy (EM).

Materials and Methods

Animals

Adult male ($N = 7$) and female ($N = 25$) Sprague Dawley rats (275–300 gm) were obtained from Taconic (Germantown, NY) and housed in groups of three with *ad libitum* access to food and water. All methods were approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee and conform to National Institutes of Health guidelines.

Phases of the estrous cycle (diestrus, proestrus, and estrus) were determined using daily vaginal smears, and cycles were followed for at least 2 weeks. Only rats with normal 4 d cycles were considered in the study. For estrogen replacement studies, ovaries were removed from female rats under isofluorane anesthesia (2% in 100% O_2), using aseptic technique. Two weeks after the surgery, rats in the estrogen-replaced group (OVX + E) were injected subcutaneously with estradiol benzoate (10 μg) suspended in 0.2 ml of sesame oil (one time per day for 3 d). Rats in the control group (OVX + O) received similar injections of sesame oil only.

Antiserum

A polyclonal rabbit antibody against phospho-Akt (Thr308) was purchased from Cell Signaling Technology (CST; Beverly, MA). Specificity of the antibody was determined using Western blot analysis. This antibody does not detect nonphosphorylated Akt or Akt phosphorylated at other sites; it also does not cross-react with related family members such as PKC or p70 S6 kinase.

To confirm the pAkt immunolocalization, a second rabbit polyclonal antiserum to pAkt [anti-phospho-Akt (Thr308); catalog number 06–678] was purchased from Upstate Biotechnology (Waltham, MA). With immunoperoxidase, the topographical distribution of the pAkt-I in the hippocampal formation was similar to that observed using the CST antiserum (data not shown).

The pAkt antiserum dilutions for quantitative light microscopy were determined on the basis of criteria described by Reis et al. (1982). Serial dilutions of the pAkt antisera established that labeling intensity was a linear function of antiserum concentration. For quantitative purposes, a dilution of 1:1000 that produced slightly less than half-maximal labeling intensity was chosen to optimize the detection of intensity variations in either direction. Similar methods have been used in several other quantitative studies (Auchus and Pickel, 1992; Pierce et al., 1999; Chang et al., 2000).

Tissue preparation

Rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused through the ascending aorta sequentially with solutions of the following: (1) 10–15 ml of physiological saline (0.9% NaCl) containing 1000 IU/ml heparin; (2) 50 ml of 3.75% acrolein (Polysciences, Warrington, PA) and 2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4; and (3) 200 ml of 2% paraformaldehyde in PB. The region of the forebrain containing the hippocampal formation was removed and cut into a 5-mm-thick coronal block and postfixed in 2% paraformaldehyde for 30 min. Sections (40 μm thick) through the hippocampal formation were cut on a Vibratome (VT1000S; Leica, Nussloch, Germany) and collected in PB. Sections from each rat were marked and divided into sets consisting of either (1) a male, proestrous, diestrous 1, and estrous females or (2) OVX + E and OVX + O. To maximize uniformity of immunocytochemical labeling conditions for the purposes of quantification, sections from each set of animals were pooled and processed together. Previous studies (Auchus and Pickel, 1992; Pierce et al., 1999) have found that this procedure eliminates differences in labeling attributable to differences in the procedure being performed on different days. Sets of the free-floating sections were treated with 1% sodium borohydride in PB before immunocytochemical labeling.

Light and electron microscopic labeling of pAkt

Peroxidase labeling. Tissue was processed for the immunocytochemical localization of pAkt according to the avidin–biotin complex (ABC) procedure (Hsu et al., 1981). Briefly, sets of sections were incubated in (1) 0.5% bovine serum albumin (BSA) in 0.1 M Tris-saline (TS), pH 7.6, for 30 min; (2) anti-pAkt antiserum (dilutions ranging from 1:500–1:2000) in 0.1% BSA in TS for 1 d at room temperature and 1 d at 4°C; (3) biotinylated goat anti-rabbit IgG (1:400; Jackson Laboratories, Bar Harbor, ME) for 30 min; (4) peroxidase–avidin complex (at twice the recommended dilution; Vector Laboratories, Burlingame, CA) for 30 min; and (5) diaminobenzidine (Aldrich, Milwaukee, WI) and H_2O_2 for 6 min.

Sections prepared for LM were mounted on gelatin-coated slides, air dried, dehydrated, and coverslipped with DPX mounting medium (Aldrich, Milwaukee, WI). Slides were examined and photographed using a Cool-Snap camera (Photometrix) attached to a Nikon Microphot microscope. Sections for EM were embedded in EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA), and ultrathin sections through the hippocampal CA1 region were prepared as described previously (Milner and Veznedaroglu, 1992). Final preparations were analyzed on a Philips CM10 electron microscope equipped with an Advanced Microscopy Techniques digital camera. Final photomicrographs were generated from digital images with a Macintosh computer 8500/120 using Adobe Photoshop 6.0 (Adobe Systems) and Quark X-Press 4.1.

Immunogold labeling. For quantitative EM studies, tissue was processed using the method of Chan et al. (1990). Briefly, sections were incubated in a 1:200 dilution of rabbit anti-pAkt in 0.1% BSA/TS for 1 d at room temperature and 1 d at 4°C. The tissue then was washed in TS, followed by a PBS, pH 7.4, wash and incubated with goat anti-rabbit IgG conjugated to 1 nm gold particles (AuroProbe One; Amersham, Arlington Heights, IL) in 0.001% gelatin and 0.08% BSA in PBS for 2 hr at room temperature. Sections were rinsed in PBS, postfixed in 1.25% glutaraldehyde in PBS for 10 min, and rinsed in PBS and 0.2 M sodium citrate, pH 7.4. The conjugated gold particles were enhanced by treatment with silver solution (IntenSE; Amersham) for 6.5–7 min. Sections were processed for EM as described above.

Data analysis

Quantitative LM. For quantitative comparisons of labeling density, hippocampal sections were measured as described previously (Chang et al., 2000). In brief, the intensity of pAkt-I in the hippocampal CA1 region was measured on a Nikon Labophot microscope in five groups of animals, each group consisting of one male and three females with each representing a phase of the estrous cycle. Quantitative densitometry, measured in pixel density units (PDUs) and performed with NIH Image, was used to assess the intensity in labeling in each lamina. Each animal was analyzed at the septal, midseptotemporal, and temporal levels of the dorsal hippocampus (three sections per animal), which corresponded to levels 31, 34, and 37 of Swanson (1992). Background staining was determined from measurements taken from white matter. Measurements were normalized by subtracting background levels before comparison. Statistical analysis was performed using either an ANOVA or *t* test; significance was accepted at $p < 0.05$.

Quantitative EM. The subcellular distribution of pAkt-immunogold (pAkt-IG) labeling in the midseptotemporal portion of the hippocampal CA1 region [corresponding to level 34 of Swanson (1992)] was compared among three sets of animals ($N = 4$ per set). For this, ultrathin sections (all with a 70 nm thickness) were cut on a UCT Ultratome (Leica) from one block per animal ($n = 12$). In each section, the labeling was quantitatively compared in portions of the tissue that were taken from a depth of 0.2–1.5 μm from the plastic interface. Only blocks that were thin-sectioned evenly across the plastic–tissue interface were included in the analysis.

Within stratum radiatum of the CA1 region, a field of 9632 μm^2 per block (25 nonoverlapping micrographs from three separate grid squares) 0.2–1.5 μm from the plastic–tissue interface was photographed at a magnification of 5800×. Structures were classified according to the definitions of Peters et al. (1991). Labeled dendrites were identified, counted, and measured for minimum diameter and area using MCID M4 Image

analysis software (Imaging Research, St. Catherine, Ontario, Canada). All pAkt-IG particles associated with cytoplasm, plasmalemma, dendritic spines, endoplasmic reticula, polyribosomes, and mitochondria were counted manually by an investigator "blind" to the experimental condition. To normalize counts in dendritic shafts, the number of pAkt-IG particles was expressed as a function of cytoplasmic area and perimeter of the plasma membrane. The number of labeled dendritic spines was expressed per 100 μm^2 of the total area analyzed from each animal. In dendritic shaft analyses, 50 randomly selected dendritic profiles from each animal were used. Statistical analysis was performed using an unpaired *t* test; significance was accepted at $p < 0.05$.

Results

In the hippocampal formation, pAkt-I is most prominent in CA1 pyramidal neurons

By LM, pAkt-I was observed predominantly in the CA1 subfield of the hippocampal formation, whereas less was observed in the CA3 subfield (Fig. 1*A*). Much lower levels of pAkt-I were detected in the dentate gyrus. Within the CA1 region, intense pAkt labeling was associated with pyramidal cell somata and their dendrites in stratum radiatum (Fig. 1*B*, *sr*). Diffuse pAkt-I also was noticeable in stratum lacunosum-moleculare (SLM) of CA1 (Fig. 1*A*, *slm*).

Qualitative EM analysis of the CA1 region in immunoperoxidase-labeled sections confirmed that pAkt-I was prominently associated with the cell bodies, but not the nuclei, of neurons in the pyramidal cell layer (PCL) (data not shown) and with dendritic profiles in stratum radiatum (Fig. 1*C*,*D*). Within dendrites, pAkt-I was found throughout the shafts and in select spines (Fig. 1*C*). However, in some cases, spines emanating from pAkt-labeled dendritic shafts appeared unlabeled (Fig. 1*D*).

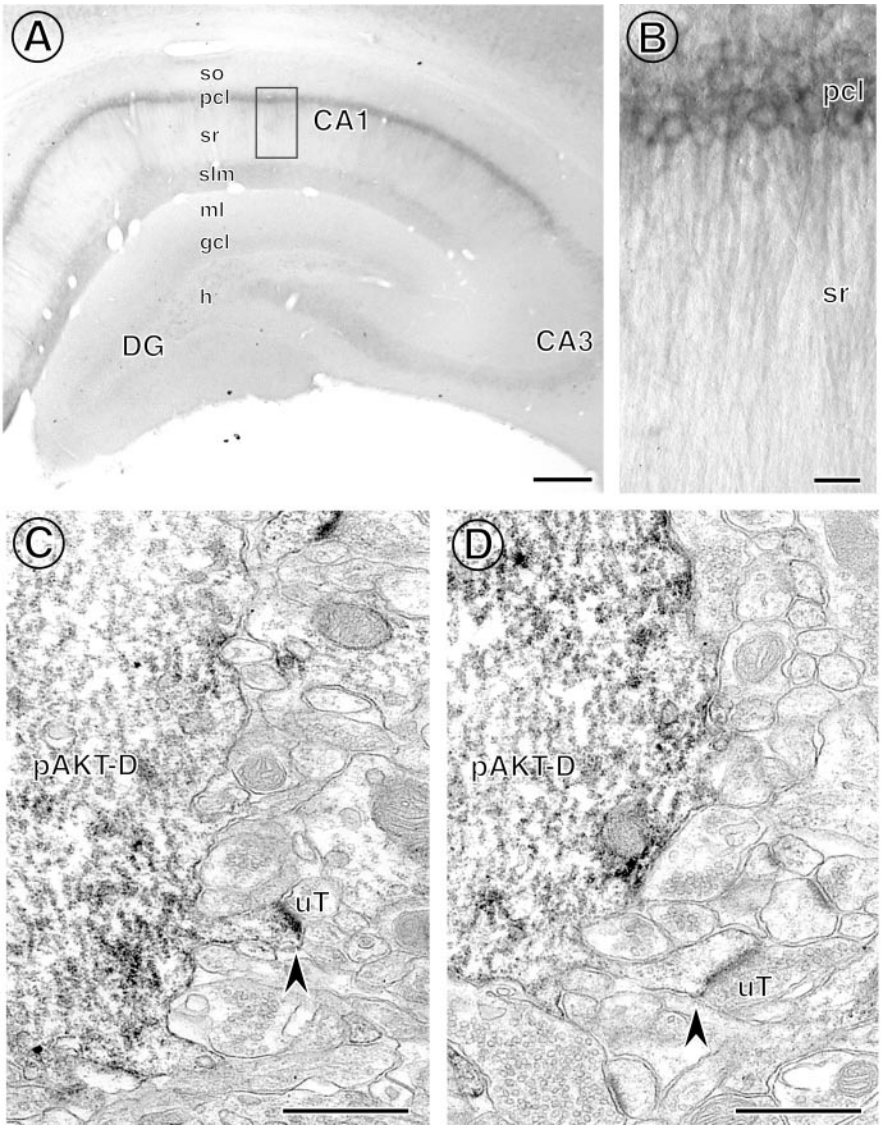

Figure 1. By LM and EM, pAkt-I is prominent in dendritic processes within stratum radiatum of the hippocampal CA1 region. *A*, Low magnification LM photomicrograph shows the distribution of pAkt-I (as demonstrated by peroxidase) in a coronal section through the dorsal rat hippocampal formation [corresponds to level 34 of Swanson (1992)]. *B*, Higher magnification of the *boxed region* in *A* shows that in the CA1 region pAkt-I is concentrated around somata in the pyramidal cell layer (*pcl*) and in dendritic processes radiating through stratum radiatum (*sr*). *C*, By EM, pAkt-I is found throughout most large dendritic shafts (*pAKT-D*) and in some dendritic spines (*arrowheads*), contacted by unlabeled terminals (*uT*). *D*, Some dendritic spines do not contain pAkt-I (*arrowhead*). *DG*, Dentate gyrus; *gcl*, granule cell layer; *h*, hilus; *ml*, molecular layer; *slm*, stratum lacunosum-moleculare; *so*, stratum oriens. Scale bars: *A*, 1 mm; *B*, 100 μm; *C*, *D*, 50 μm.

The density of pAkt-I in stratum radiatum is greatest at proestrus

To determine whether the levels of circulating steroids across the estrous cycle and in males influence the expression of pAkt-I in stratum radiatum of CA1, LM quantitative densitometry was used. Densitometric analysis revealed that the intensity of pAkt-I in SR was significantly greater in proestrus rats compared with diestrus and male rats (ANOVA: pro-estrus/diestrus, $p = 0.02$; proestrus/male, $p = 0.0003$) (Fig. 2). A similar trend was seen in the PCL, SLM, and dorsal blade of the dentate molecular layer (ML); however, statistical significance was found only between proestrus female rats and male rats in these regions (ANOVA; PCL, $p = 0.005$; SLM, $p = 0.006$; ML, $p = 0.03$).

Consistent with these findings, increased pAkt-I was observed in SR of CA1 in OVX + E rats compared with OVX + O [OVX + E: 17.4 ± 0.9 PDU ($N = 5$); OVX + O: 12.6 ± 0.5 PDU ($N = 5$); *t* test; $p < 0.0001$). Significantly higher densities of pAkt-I in OVX + E compared with OVX + O rats also were observed in the PCL (OVX + E: 30.0 ± 1.4; OVX + O: 25.2 ± 1; *t* test; $p = 0.002$), SLM (OVX + E: 17.6 ± 0.8; OVX + O: 14.1 ± 0.7; *t* test; $p = 0.002$), and dentate ML (OVX + E: 8.6 ± 0.3; OVX + O: 7.3 ± 0.4; *t* test; $p = 0.009$).

pAkt-I increases in dendritic spines at proestrus

To determine whether the changes in stratum radiatum pAkt-I densities by LM across the estrous cycle and between females and males occur within particular cellular compartments, pAkt-IG-labeled sections from three sets of rats were examined by EM. In agreement with the immunoperoxidase localization of pAkt, pAkt-I identified by immunogold was predominantly in dendritic profiles (Figs. 3*A–C*, 4*A*, 5*A*, 6*A*). Moreover, pAkt-IG

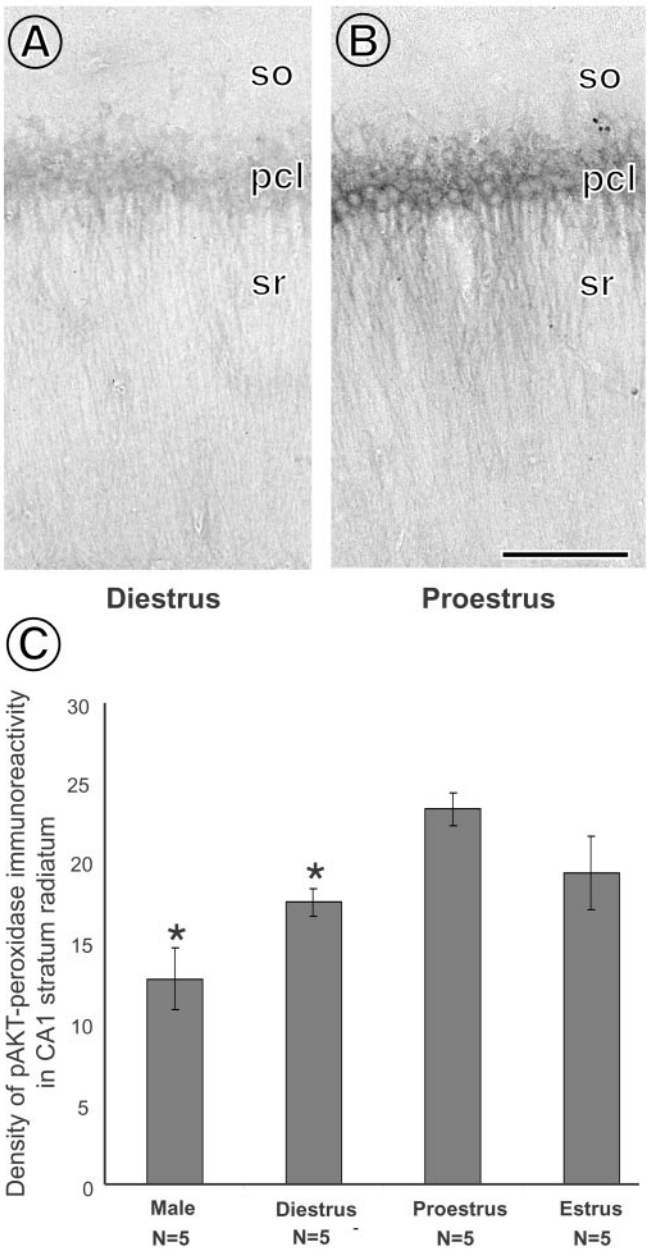

Figure 2. The density of pAkt-I in CA1 stratum radiatum is highest in proestrus rats. Representative LM micrographs showing peroxidase pAkt labeling in the CA1 region of a coronal hippocampal section from a diestrus (*A*) and proestrus (*B*) rat. *C*, The density of pAkt-I (measured in pixel density units) in CA1 stratum radiatum differs between female rats across estrus and male rats. *Asterisk* indicates significant differences from proestrus (ANOVA; Fisher's *post hoc*). *pcl*, Pyramidal cell layer; *so*, stratum oriens; *sr*, stratum radiatum. *N*, Number of animals per condition. Scale bar: (in *B*) *A*, *B*, 100 μm.

could be discretely localized to the spine apparatus (Fig. 3*A*,*B*), the plasma membrane (Fig. 3*C*) of dendritic spines, and the smooth endoplasmic reticula or plasma membrane of dendritic shafts (Figs. 4*B* or 6*A*, respectively). Dendritic shafts and spines containing pAkt labeling appeared morphologically similar between male and female rats.

Quantitative EM analysis revealed an almost a sixfold increase in the number of pAkt-IG particles associated with the dendritic spines (both cytoplasmic and plasma membrane) of proestrus rats compared with diestrus and estrus rats (*t* test; $p < 0.0001$) and an approximately threefold increase in the number of pAkt-IG particles associated with dendritic spines in proestrus female rats compared with male rats (*t* test; $p < 0.0001$) (Fig. 3*D*). To address the possibility that the increase in pAkt-IG labeling simply reflects a scaling up with the overall increases in spine number that occur in the proestrus phase of the estrous cycle (Woolley, 1998, 1999), the ratio of spines containing pAkt-IG particles to the total number of spines in the field was examined (Fig. 3*E*). This analysis demonstrated that the proportion of pAkt-IG-labeled spines was significantly higher in proestrus rats than in either diestrus or estrus or male rats (*t* test; $p < 0.0001$; for all) and that the changes in the number of gold particles reflects changes in the number of labeled spines (Fig. 3*D*).

In dendritic shafts, significantly more pAkt-IG particles were associated with endoplasmic reticula (Fig. 4*A*) and structures resembling polyribosomes (Steward and Falk, 1991) in proestrus rats (Fig. 4*B*) than in diestrus, estrus, and male rats (*t* test; $p < 0.0001$, $p = 0.0005$, and $p < 0.0001$, respectively). Additionally, a significantly higher number of pAkt-IG particles were located within 0.1 μm of the base of dendritic spines in proestrus rats compared with diestrus, estrus, and male rats (*t* test; $p < 0.0001$, $p = 0.0002$, and $p = 0.006$, respectively) (Fig. 5*A*,*B*). pAkt-IG particles at the base of dendritic spines were often affiliated with the spine apparatus (Fig. 5*A*).

To determine whether the increases in pAkt-I seen at the light level were a result of increased levels of pAkt-I within dendritic shafts, the total number of pAkt-IG particles was determined. The total number (i.e., cytoplasm + plasma membrane) of pAkt-IG particles per square micrometer of dendritic shaft profiles was not significantly different across estrous females and in males [proestrus ($n = 150$): $0.30 + 0.01/\mu m^2$; diestrus ($n = 150$): $0.30 + 0.1/\mu m^2$; estrus ($n = 150$): $0.30 + 0.1/\mu m^2$; male ($n = 150$): $0.27 + 0.01/\mu m^2$; $p > 0.05$]. However, when the distribution of pAkt-IG particles on the plasma membrane and within the cytoplasm was analyzed separately, proestrous rats had a significantly higher number of pAkt-IG particles on the plasmalemma (*t* test; $p < 0.0001$) compared with diestrous rats (Fig. 6*B*). No significant differences between the groups (*t* test; $p = 0.05$) were found in the number of gold particles within cytoplasm (Fig. 6*C*). These results show that pAkt-IG particles redistribute, without changing in number in dendritic shafts, in response to fluctuating ovarian hormone levels.

Discussion

This study demonstrates that proestrus rats compared with diestrus, estrus, and male rats contained a significantly higher number of pAkt-IG particles associated with (1) dendritic spines (both the cytoplasm and plasma membrane), (2) spine apparati located within 0.1 μm of dendritic spine bases, (3) endoplasmic reticula and polyribosomes in the cytoplasm of dendritic shafts, and (4) the plasma membrane of dendritic shafts. Because previous studies have shown that both brain estrogen levels and synaptogenesis are elevated during the proestrus phase of the cycle (Gibbs, 1996; Woolley, 1998), the estrogen-dependent alteration of pAkt-I distribution within dendrites suggests a possible role for pAkt in local events associated with synaptic formation and breakdown. This includes the possibility of estrogen-regulated spine synapse formation by signaling transduction via Akt. At the same time, pAkt-I is increased in the somata of CA1 pyramidal cells after estrogen treatment or during proestrus, suggesting that other aspects of intracellular signaling through Akt may also be regulated by estrogens.

Methodological considerations

The present study discovered differences in pAkt-I at the light microscopic level by comparing densitometric measurements of immunoperoxidase reaction product. When sufficiently high dilutions of primary antisera are used, this method has proven reliable for quantifying differences in immunolabeling across experimental conditions (Pierce et al., 1999; Chang et al., 2000). For electron microscopic comparisons of pAkt-I, the pre-embedding immunogold method was used. This method provides discrete subcellular localization of reaction product while maintaining morphological preservation (Leranth and Pickel, 1989) and is more suitable than postembedding methods for localization of immunoreactivity at extrasynaptic sites and for determining regional distributions (Lujan et al., 1996). However, the localization of pAkt-I using the pre-embedding immunogold method is likely to provide underestimates because of the more limited penetration and sensitivity of this method compared with the immunoperoxidase technique (Leranth and Pickel, 1989). These factors would not likely influence relative comparisons between groups because tissue from each group was processed together using identical experimental conditions, and ultrathin sections were always collected adjacent to the exposed surface of tissue, where access to immunoreagents is most complete.

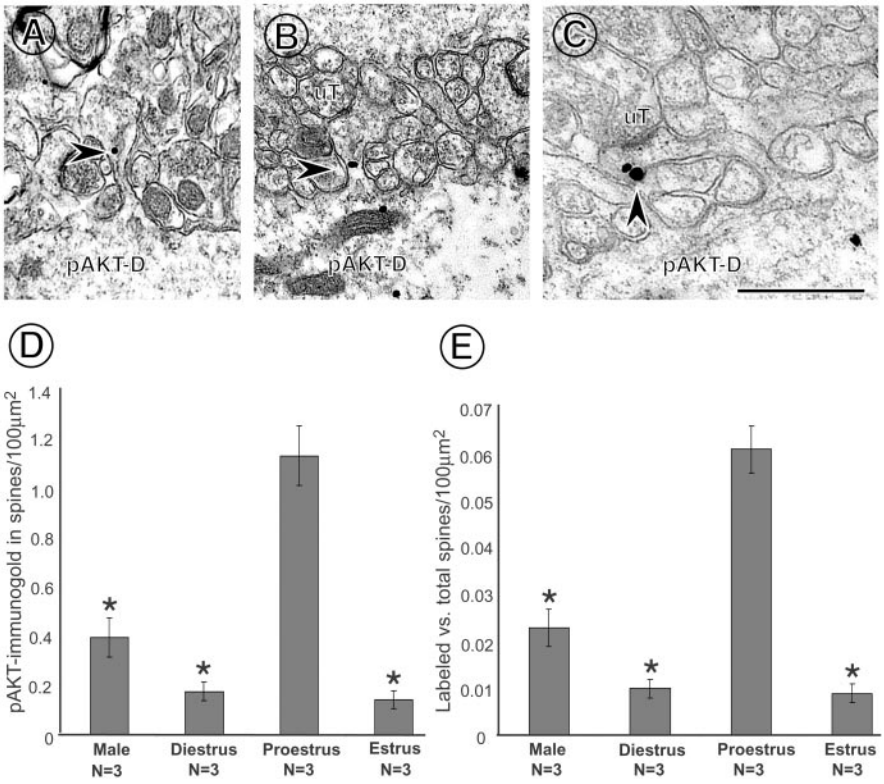

Figure 3. By EM, pAkt-IG labeling associated with dendritic spines is significantly higher in proestrus rats. *A–C*, Representative electron micrographs showing the distribution of pAkt-IG particles (*arrowheads*) associated with the spine apparatus (*A, B*) or the plasma membrane (*C*) of dendritic spines. Unlabeled terminals (*uT*) contact both labeled spines. In *B*, the labeled spine arises from a pAkt-IG-labeled dendritic shaft (*pAKT-D*). *D*, The total number of pAkt-IG particles in dendritic spines (number of pAkt-IG particles associated with both cytoplasm and plasma membrane of spines per 100 μm^2 of tissue) is significantly higher in proestrus rats. *E*, The relative number of labeled spines is highest in proestrus. The relative number of labeled spines was calculated as the number of dendritic spines containing pAkt-IG particles (i.e., pAkt-labeled) divided by the total number of dendritic spines (i.e., labeled + unlabeled) as represented per 100 μm^2 of the total field of 9632 μm^2 for each rat. *Asterisk* indicates significant differences from proestrus (unpaired *t* test). *N*, Number of rats per condition. Scale bar: (in *C*) *A*, *B*, *C*, 50 μm.

Estrogens increase pAkt-I in pyramidal cell bodies

By light microscopy, the intensity of pAkt-I in the pyramidal cell layer was highest in proestrous or OVX rats with estradiol supplements. Estrogen-mediated stimulation of PI3-K and the subsequent phosphorylation of pAkt in cell bodies may be linked to a wide array of signaling effects, including neuroprotection, cell proliferation, metabolic regulation, differentiation, and protein synthesis (Downward, 1998). For example, Akt may affect neuronal survival via translational regulation in both transcription-dependent and independent manners (Brunet et al., 2001). Although future studies could address such effects within the neuronal cell body, this study focuses on the role of pAkt-I at the dendritic membranes and how Akt may participate in estrogen-mediated synaptogenesis.

Estrogens may induce pAkt translocation to dendritic membranes

By light microscopy, the density of pAkt-I in stratum radiatum of CA1 was highest in proestrus rats and OVX rats supplemented with estradiol benzoate. Moreover, by electron microscopy, the number of pAkt-IG particles on the plasmalemma of dendritic profiles was increased in proestrus rats (although the total number of pAkt-IG particles in dendritic shafts was not changed). Akt is known to translocate to the plasma membrane after association of its pleckstrin homology domain with lipids produced by PI3-K; such steps are necessary for presentation of Akt to upstream activating kinases (Hemmings, 1997; Downward, 2001). Thus, the membrane association of the activated form of Akt (pAkt) could localize the signaling intermediate at the dendritic spine and position it for direct participation in synaptogenesis.

Putative pAkt involvement in estrogen-regulated synaptogenesis

During proestrus, pAkt-I is significantly increased in dendritic spines but not dendritic shafts in stratum radiatum of the CA1 region. Previous studies have shown that both brain estrogen levels and synaptogenesis are highest during this phase of the cycle (Gibbs, 1996; Woolley, 1998). Moreover, several synaptic markers (synaptophysin, syntaxin, and spinophilin) are increased when estrogen levels are highest (Brake et al., 2001). Together, these findings suggest that estrogens might activate intracellular signaling cascades locally within dendritic spines. Significantly, pAkt has been shown in other systems to activate downstream translational mechanisms, e.g., FKBP-12-rapamycin-associated protein (FRAP)/mammalian target of rapamycin (mTOR), 4E-BP1, and p70S6-kinase, to regulate protein translation (Burgering and Coffer, 1995; Gingras et al., 1998). Moreover, a recent study indicated that essential components of the pathway downstream of pAkt, including 4E-BP1, are located in dendrites of the hippocampal CA1 region (Tang et al., 2002). The alteration that we report in the subcellular distribution of pAkt-I within the cytoplasm of dendritic spines and shafts with varying estrogen levels is consistent with a role for pAkt in

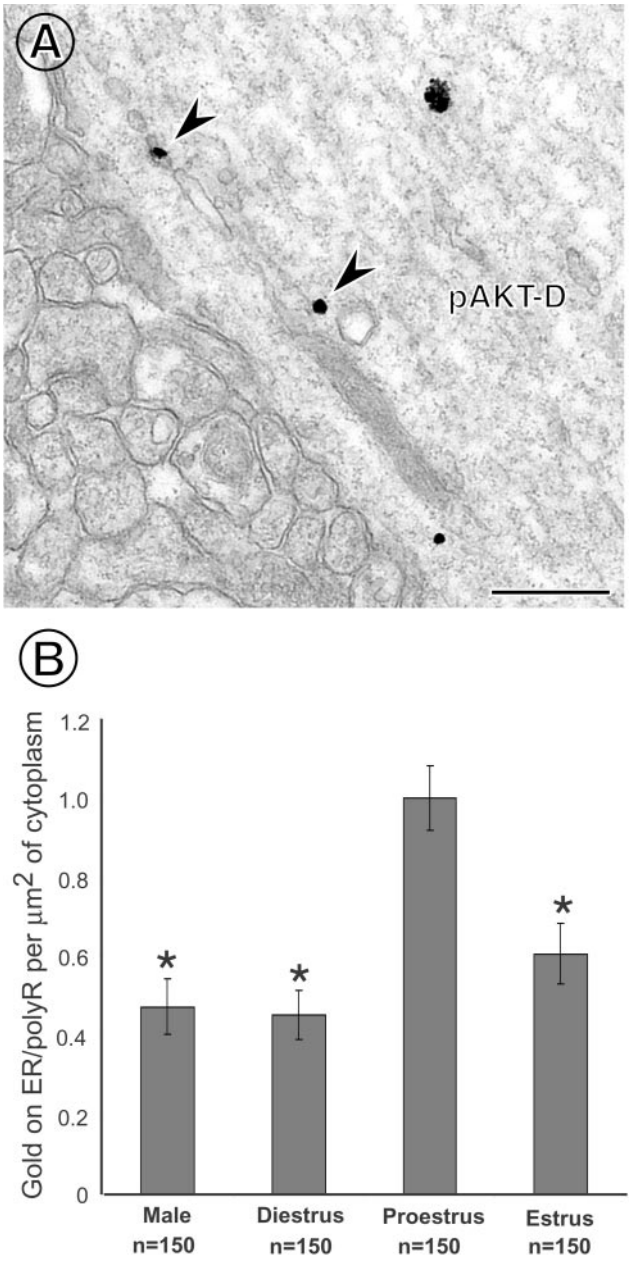

Figure 4. In dendritic shafts, pAkt-IG labeling associated with endoplasmic reticula and polyribosomes is highest in proestrus rats. *A*, Representative electron micrograph showing pAkt-IG particles associated with endoplasmic reticula (*arrowheads*) of a pAkt-IG-containing dendritic shaft profile (*pAKT-D*). *B*, Variations in the number of pAkt-IG particles per 1 μm^2 of dendritic area that are associated with endoplasmic reticula and polyribosomes in dendritic shafts of female rats across estrous and male rats. *Asterisk* indicates significant differences (unpaired *t* test) from proestrus rats. n = number of dendrites analyzed from three rats per condition. Scale bar, 50 μm.

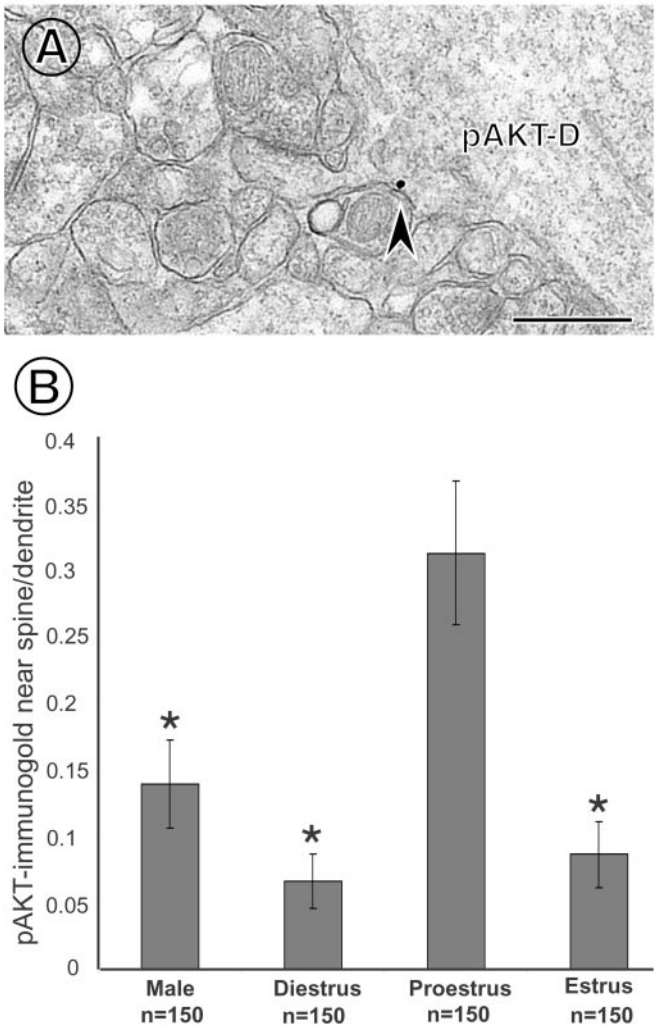

Figure 5. pAkt-IG labeling near the base of dendritic spines is highest in proestrus rats. *A*, Representative electron micrograph showing a pAkt-IG particle (*arrowhead*) affiliated with a portion of the spine apparatus located within 0.1 μm from the base of a dendritic spine. *B*, Variations in the number of pAkt-IG particles within 0.1 μm of the bases of dendritic spines in female rats across estrous and male rats, calculated per dendrite. *Asterisk* indicates significant differences from proestrus (unpaired *t* test). n = number of dendrites analyzed from three rats. Scale bar, 50 μm.

the local protein translation events responsible for synaptic formation and breakdown associated with the estrous cycle.

Involvement of pAkt in estrogen-mediated signaling events

The present demonstration that estrous cycle-dependent pAkt-I is found in dendritic spines, together with our previous findings that ERα immunoreactivity is also in dendritic spines, supports a mechanism whereby estrogens regulate local signal transduction *in vivo* of the Akt/4E-BP1 pathway. There is a growing body of evidence that estrogens produce rapid effects at membranes via signal transduction intermediates, including those coupled with G-protein receptors (Kelly and Wagner, 1999; Kelly et al., 1999). *In vitro*, estrogens can rapidly regulate gene expression through signal transduction intermediates including Akt and MAPK (Kelly and Levin, 2001). Moreover, estrogens can increase intracellular Ca^{2+} concentrations, which activates neuronal Akt *in vitro* (Yano et al., 1998), and activate adenylate cyclase, stimulating PKA and PKC pathways (Levin, 2001). Estrogen activation of MAPKs and the PI3-K pathway leads to stimulation of the Akt pathway (Toran-Allerand et al., 1999; Honda et al., 2000; Kelly and Levin 2001; Belcher and Zsarnovszky, 2001). Importantly, these events appear to be mediated through ERα and not ERβ (Simoncini et al., 2000). *In vivo*, changes in hippocampal synaptic structure and function in response to estrogen have been correlated with alterations in glutamatergic receptor activity. Specifically, estrogen treatment increases the synthesis of the NMDA receptor subunit NR1 and agonist binding to the NMDA receptor (Gazzaley et al., 1996; Woolley et al., 1997). Estrogens can rapidly potentiate kainate-induced currents (Gu and Moss, 1996) and

enhance NMDA receptor-mediated EPSPs and long-term potentiation (LTP) (Foy et al., 1999). Estrogen-mediated activation of the extracellular regulated kinase (ERK)/MAPK pathway in the hippocampus is involved in the rapid effects of estrogen on NMDA receptors and LTP through tyrosine phosphorylation of NMDA receptor NR2 subunits (Bi et al., 2000). In particular, cyclic changes in estrogen levels during the estrous cycle are associated with corresponding changes in the levels of activation of ERK2 and the phosphorylation state of the NR2 subunits (Bi et al., 2001). On the basis of *in vitro* evidence (Belcher and Zsarnovszky, 2001; Kelly and Levin 2001), these estrogen-stimulated modifications of excitatory glutamatergic synapses also are likely to involve Akt *in vivo*.

pAKT may be involved in local protein synthesis

Synapses that undergo changes in strength are in need of newly synthesized proteins; in addition to transporting new proteins from the soma, another possible mechanism is local protein synthesis regulated by synaptic activation (Steward and Schuman, 2001). Synapse-associated polyribosome complexes and associated membranous cisterns are located beneath postsynaptic sites on dendrites and often are associated with or near the base of a spine (Steward and Fass, 1983). The present study shows increased pAkt-I associated with endoplasmic reticula and polyribosomes in the dendritic profiles in proestrus, rats supporting the existence of a translational machinery specifically designed to act on dendrite-localized mRNAs for synaptic components. Moreover, our studies *in vitro* demonstrate that estrogen can drive the neuronal synthesis of PSD-95, a protein that is important in spine maturation (El-Husseini et al., 2000), in an Akt-dependent manner (Akama and McEwen, 2003). Immunoreactivity to P-proteins of large ribosomal subunits and to S3-proteins of small ribosomal subunits associated with membranes increases in dendritic shafts and spines preceding the period of estrogen-induced synaptogenesis (J. B. McCarthy and T. A. Milner, unpublished observations).

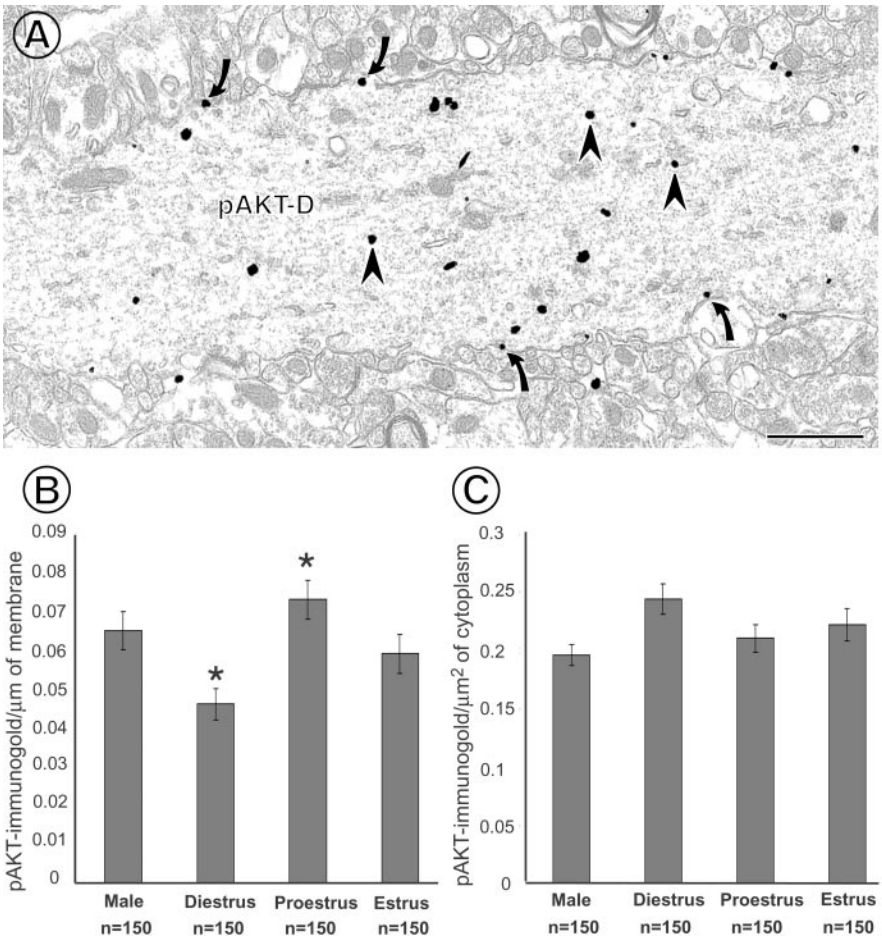

Figure 6. The number of pAkt-IG particles on the plasma membrane of dendritic shafts is highest in proestrus rats. *A*, Representative electron micrograph of a dendritic shaft profile showing pAkt-IG particles in the cytoplasm (*arrows*) and on the plasma membrane (*arrowheads*). *B*, The total number of pAkt-IG particles per 1 μm of dendritic plasma membrane of dendritic shafts is significantly different in proestrus rats compared with disestrus rats (unpaired *t* test; $*p < 0.05$). *C*, Cytoplasmic pAkt-IG particles in dendritic shafts (expressed per 1 μm^2) are not significantly different between female rats across estrous and male rats. $n =$ number of dendrites analyzed from three rats per condition. Scale bar, 50 μm.

In conclusion, our findings that the highest levels of estrous cycle-dependent pAkt-I in dendritic spines are observed when dendritic spine and synapse formation also are highest supports a mechanism whereby estrogens regulate local signal transduction *in vivo*, via the Akt/4E-BP1 pathway. Such a localized mechanism responsive to estrogen would be instrumental to the rapid, hormone-regulated cyclic formation of synapses that occurs in the CA1 region of the hippocampus.

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