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EFFECTS OF ESTROGEN AND AGING ON THE SYNAPTIC DISTRIBUTION OF PHOSPHORYLATED AKT-IMMUNOREACTIVITY IN THE CA1 REGION OF THE FEMALE RAT HIPPOCAMPUS

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Abstract

The estrogen 17 β -estradiol (E) increases the axospinous synaptic density and plasticity in the hippocampal CA1 region of young female rats but fails to do so in aged female rats. This E stimulus on synaptic plasticity is associated with the phosphorylation-dependent activation of Akt kinase. Our previous findings demonstrated that increased estrogen levels subsequently increase phosphorylated Akt (pAkt)-immunoreactivity (-IR) within the dendritic shafts and spines of pyramidal neurons in young female rats. Therefore, because Akt can promote cell survival and growth, we tested the hypothesis that the less plastic synapses of aged female rats would contain less E-stimulated pAkt-IR. Here, young (3–4 months) and aged (22–23 months) female rats were ovariectomized seven days prior to a 48-hour administration of either vehicle or E. The pAkt-IR synaptic distribution was then analyzed using post-embedding electron microscopy. In both young and aged rats, pAkt-IR was found in dendritic spines and terminals, and pAkt-IR was particularly abundant at the post-synaptic density. Quantitative analyses revealed that the percentage of pAkt-labeled synapses was significantly greater in young rats compared to aged rats. Nonetheless, E treatment significantly increased pAkt-IR in pre- and post-synaptic profiles of both young and aged rats, although the stimulus in young rats was notably more widespread. These data support the evidence that hormone-activated signaling associated with cell growth and survival is diminished in the aged brain. However, the observation that E can still increase pAkt-IR in aged synapses presents this signaling component as a candidate target for hormone replacement therapies.

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INTRODUCTION

In the young female rat hippocampus, increasing estrogen levels consequently increase the dendritic spine density and synapse number in the CA1 region of stratum radiatum (Gould et al., 1990; Woolley and McEwen, 1992; Woolley et al., 1996). However, estrogen fails to increase this synaptic density in the same hippocampal region in aged female rats (Adams et al., 2001c). As these morphologic alterations to synaptic plasticity are tightly coordinated by signal transduction pathways contained within the dendritic shafts and spines (Flavell and Greenberg, 2008; Newpher and Ehlers, 2009), it is necessary to consider how estrogens might nongenomically impact the signaling pathways that regulate spine growth and spine morphology and then determine if this non-genomic effect is diminished in the aged brain.

We demonstrated previously that estrogen can stimulate the non-genomic, phosphorylation-dependent activation of several signal transduction pathways associated with synaptic plasticity, including the LIM kinase (LIMK) pathway (Kramar et al., 2009; Yildirim et al., 2008), the cAMP response element binding protein (CREB) pathway (Lee et al., 2004), and the phosphatidylinositol-3-kinase (PI3K) / Akt (protein kinase B) pathway (Akama and McEwen, 2003). Akt in particular is a key signal transduction intermediate that is relevant to alterations in synaptic morphology as Akt promotes synaptic plasticity, cell survival, and protein translation-related cell growth (Brunet et al., 2001; Chong et al., 2005; Mannella and Brinton, 2006; Ruggero and Sonenberg, 2005; Titolo et al., 2008; Wang et al., 2003).

Akt is a protein kinase that is activated by the phosphorylation of specific residues (Downward, 1998; Woodgett, 2005). *In vitro*, the estrogen 17 β -estradiol (E) can activate PI3K which subsequently results in such downstream Akt phosphorylation and activation (Akama and McEwen, 2003; Mannella and Brinton, 2006). Furthermore, E can stimulate the protein translation of the dendritic spine protein post-synaptic density (PSD)-95 via estrogen receptor- α (ER α) and the PI3K/Akt pathway (Akama and McEwen, 2003). In young mice and rats *in vivo*, elevated estrogen levels [elevated either endogenously during proestrus or exogenously in ovariectomized (OVX) animals with E replacement] increases the level of phosphorylated Akt (pAkt)-immunoreactivity (-IR) in CA1 stratum radiatum (Spencer et al., 2008; Znamensky et al., 2003). Detailed analyses at the ultrastructural level further demonstrate that this estrogen-stimulated pAkt-IR is localized to dendritic spines, the spine apparatus, the endoplasmic reticulum in dendritic shafts, and the plasmalemma of dendritic spines (Znamensky et al., 2003). Thus, these findings suggest that the localized activation of the Akt pathway is well positioned to facilitate the rapid, estrogen-stimulated increase in spine density and synaptogenesis observed in the CA1 region of the hippocampus.

Because Akt plays a critical role in cell growth and in PSD-95 protein translation (Akama and McEwen, 2003), we tested the hypothesis that the less plastic CA1 synapses of aged female rats may contain diminished levels of E-stimulated pAkt-IR, as is the case for pLIMK-IR (Yildirim et al., 2008). We therefore sought to determine if the level or the synaptic distribution of E-stimulated pAkt-IR is affected by aging. To address this question, the sub-cellular distribution of pAkt-IR in the hippocampal CA1 region was analyzed in young (3-4 months old) vs. old (22-23 months old) OVX female rats that were administered either E or vehicle. Post-embedding electron microscopy (EM) was employed as this high-power magnification approach can render quantifiable differences in pAkt-IR within discrete, partitioned sub-regions of the dendritic spine compartment and the synapse complex (Adams et al., 2002; Yildirim et al., 2008).

RESULTS

Post-embedding immunocytochemistry was performed to examine quantitatively if age and/or E affect the levels or subcellular distribution of pAkt-IR in the CA1 stratum radiatum of the dorsal hippocampus. Young and aged female rats were OVX 7 days prior to implanting silastic capsules containing either vehicle (cholesterol) or E (10% in cholesterol). Two days after the implant, the animals were perfusion fixed with glutaraldehyde and paraformaldehyde, and the dorsal hippocampus was embedded in Lowicryl. Sections through CA1 stratum radiatum were cut on an ultratome, collected onto grids and labeled with antibodies to pAkt. Immunoreactivity for pAkt was identified using gold-conjugated secondary antibodies. The sections then were examined under an electron microscope and the percentage of axospinous synapses labeled for pAkt as well as the numbers of immunogold particles in pre- and post-synaptic compartments were quantified (see details in Experimental Procedures).

pAkt-IR is most prominent in post-synaptic compartments

In agreement with our previous pre-embedding EM studies (Znamensky et al., 2003), pAkt-IR is found in dendrites, dendritic spines, and terminals in the CA1 stratum radiatum from all animal groups using post-embedding EM immunocytochemical methods. Additionally, post-embedding EM revealed pAkt-IR in all compartments of the synapse, including the non-synaptic component of the terminals and dendritic spines (Fig. 1). Within a given group, the synaptic distribution of pAkt-IR was more pronounced in the post-synaptic component of the synapse than in the pre-synaptic terminal and in the cleft, with prominent labeling within the non-synaptic core of the spine. Within the post-synaptic region, the distribution of pAkt immunogold particles was non-uniform in all groups. These labeling patterns were not seen in tissue processed in the absence of primary antibody or in the presence of non-gold conjugated secondary IgG.

Although synaptic pAkt-IR decreases with aging, it increases with estradiol in both young and aged rats

To determine whether synaptic pAkt-IR is affected by age and/or circulating estrogen, we first determined the overall percentage of axospinous synapses in CA1 that contained pAkt immunogold particles in young and aged OVX female rats that were administered either E or vehicle. For this, the percentage of pAkt-labeled axospinous synapses within a 3000 μm^2 area in CA1 stratum radiatum was assessed. Dendritic spines (bins 1-4; see figure 3) that contained 2 or more immunogold particles were considered labeled.

We found that both age and estrogen treatment significantly impacted the percentage of axospinous synapses that were pAkt-IR (Fig. 2). The multiple comparisons analysis using the Tukey-Kramer adjustments (Ludbrook, 1998) revealed that the young OVX + Vehicle group had 43% more pAkt-immunoreactive synapses than the aged OVX + Vehicle group ($p < 0.0002$). However, regardless of age group, OVX + E rats had significantly more pAkt-IR synapses than OVX + vehicle rats. Specifically, the young OVX + E group had 42% more pAkt-immunoreactive synapses than young OVX + vehicle group ($p < 0.0001$). Moreover, aged OVX + E group had 60% more pAkt-immunoreactive synapses than aged OVX + Vehicle group ($p < 0.0001$). The young OVX + E and aged OVX + E groups were also significantly different from each other. Specifically, the young OVX + E group had 19% more pAkt-immunoreactive synapses than the aged OVX + E group ($p < 0.03$).

The overall group effect was highly significant ($p < 0.0001$). The main contribution to the significant overall group effect was the difference between the young OVX + E and aged OVX + Vehicle groups. The age-by-treatment additive effect was significant ($p < 0.0001$),

and the percentage of pAkt-IR associated with spines decreased 68% due to the age-by-treatment interaction.

Estrogen-induced increases in pAkt-IR are more limited in synaptic complexes of aged rats compared to young rats

To determine if the changes in pAkt-IR seen in aging and following E administration were restricted to specific cellular compartments, the sub-cellular distribution of pAkt immunogold particles in synaptic complexes was counted using a SynBin analysis (see Experimental Procedure for details). For this, immunogold particles were categorized into one of eight bins contained in regions of the pre-synaptic terminal, synaptic cleft, or post-synaptic dendritic spine (Fig. 3). Regardless of age or treatment, the highest number of pAkt immunogold particles was in the post-synaptic compartment, the non-synaptic areas of the dendritic spine (Bin 4), and within the bins associated with the post-synaptic density (Bins 1 and 2). In the pre-synaptic compartment, pAkt immunogold particles were primarily found in non-synaptic areas of the terminal (Bin 8) and, to a lesser extent, in regions of the terminal adjacent to the post-synaptic profile (Bins 6 and 7). Few pAkt immunogold particles were detected synaptic cleft region (Bin 5). Almost no immunogold particles were detected the lateral to the post-synaptic density (Bin 3) and thus this bin was excluded from the analysis.

Compared to young OVX rats, aged OVX rats had fewer pAkt immunogold particles associated with bins found in both pre- and post-synaptic sites near the post-synaptic density (Fig. 4). Specifically, aged OVX females had fewer pAkt immunogold particles in the 0-30 nm post-synaptic bin (Bin 1; $p < 0.002$), 30-60 nm post-synaptic bin (Bin 2; $p < 0.001$ for Bin 2), 0-30 nm pre-synaptic bin (Bin 6; $p < 0.004$) and 30-60 nm pre-synaptic bin (Bin 7; $p < 0.005$ for Bin 7). No age-related difference in the levels of pAkt immunogold particles was observed in the cleft (Bin 5), dendritic spine, or bins representing the non-synaptic core of the spine (Bin 4) or the terminal (Bin 8).

In young rats, E significantly increased the number of pAkt immunogold particles in most compartments of the synaptic complex (Fig. 4A). Specifically, compared to young OVX rats, young OVX + E rats had significantly more pAkt immunogold particles within the 0-30 nm postsynaptic bin (Bin 1; $p < 0.0003$), the 30-60 nm post-synaptic bin (Bin 2; $p < 0.001$), the > 60 nm post-synaptic bin (Bin 4; $p < 0.001$), cleft (Bin 5; $p < 0.008$), and the > 60 nm pre-synaptic bin (Bin 8; $p < 0.0003$). The difference between control OVX and E-treated animals in the 30-60 nm pre-synaptic bin (Bin 7) was close to the limit of statistical significance ($p = 0.051$).

In the aged animals, the E-induced increases in the number of pAkt immunogold particles were more limited (Fig. 4B). Compared to OVX aged animals, OVX + E animals only had significant increases in pAkt immunogold particles in the 0-30 nm and 30-60 nm post-synaptic bins (Bin 1 and 2; $p < 0.0001$ and $p < 0.03$, respectively) and in the 0-30 nm pre-synaptic bin (Bin 6; $p < 0.05$). Moreover, unlike the young rats, aged OVX rats treated with E had significantly less pAkt immunogold particles in the >60 nm post-synaptic bin (Bin 4; $p < 0.05$).

Age and E treatment were found to have independent effects on the synaptic distribution of pAkt immunogold particles: aging decreases pAkt-IR while E treatment increases pAkt-IR. The multiple comparison analysis by Tukey-Kramer adjustments revealed that all of the bins made significant contributions to the group effect.

DISCUSSION

The ultrastructural analysis of pAkt immunogold particle distribution in CA1 and its alteration by estrogen status and/or age has led to a number of interesting and potentially important findings (Fig. 5). First, pAkt immunogold particles are present in both pre- and postsynaptic profiles, with a prominent localization in the post-synaptic density. Second, the overall percentage of axospinous synapses labeled for pAkt decreases with aging and increases following estrogen replacement in both young and aged rats. Third, estrogen-induced changes in the sub-cellular distribution of pAkt immunogold particles in the pre- and post-synaptic compartments are more limited in aged rats compared to young rats. These findings suggest the Akt pathway may be an important target for hormone replacement therapies in menopause and age-related neurologic disorders in general (Frick, 2009; Gresack and Frick, 2006).

pAkt-IR is prominent in synaptic complexes and decreases with age

The localization of pAkt-IR to terminals and dendritic spines in the rat CA1 region using post-embedding methods confirm and extend our previous study using pre-embedding EM (Znamensky et al., 2003). Regardless of age or treatment, pAkt immunogold particles were found in all regions of the pre- and post-synaptic profiles. However, pAkt immunogold particles were notably higher in the pre- and post-synaptic regions near the post-synaptic density. Compared to young OVX rats, the overall levels of pAkt-IR were significantly decreased in synaptic complexes in aged OVX rats. Further analyses of the changes in the sub-cellular distribution of pAkt immunogold particles in terminals and dendritic spines of aged and young OVX revealed that the most significant decreases in pAkt-IR occurred in the post-synaptic density and in pre-synaptic regions apposed to the post-synaptic density. These observations suggest that aging could impact transmitter levels or release and/or post-synaptic receptivity (Sanna et al., 2002; Wang et al., 2003).

The findings that pAkt-IR is reduced with aging are consistent with many of our previous studies. In particular, we have found that aged animals have lower levels of ER α associated with synaptic complexes compared to young animals (Adams et al., 2002). Moreover, we have found that pLIMK-IR is similarly diminished in the synaptic complexes of aged rats (Yildirim et al., 2008). The findings suggest that aging decreases the access or the synaptic presentation of the components necessary to coordinate or to co-activate an estrogen response at or near the postsynaptic density complex. In support of this suggestion, we have recently found that the level of the post-synaptic density marker, PSD-95, begins to decrease in the hippocampus of middle aged rats (11 months old) and is significantly decreased in the hippocampus of (24 month old) aged rats (Williams et al., to be submitted to Special issue).

Estrogen increases pAkt-IR in synaptic complexes in young and aged rats

In both young and aged OVX rats, estrogen administration increased the percentage of axospinous synapses with pAkt-IR in the CA1 region of the stratum radiatum. Furthermore, the relative percentage of these estrogen-induced changes in pAkt-labeled synaptic complexes were greater in aged rats than in young rats (60% and 42%, respectively). Additional analysis of the sub-cellular distribution of the estrogen-induced changes in pAkt-IR demonstrated that pAkt-IR increased in more synaptic regions of the young rats than in the aged rats. Specifically, following estrogen-administration, pAkt-IR was elevated in synaptic as well as cytoplasmic regions of both pre- and post-synaptic profiles in the young rats. In contrast, estrogen administration increased pAkt-IR only in the region of the post-synaptic density closest to the plasma membrane and in the cytoplasm of the pre-synaptic terminal. Moreover, estrogen administration decreased pAkt-IR in the cytoplasmic regions of the dendritic spine. Thus, these findings demonstrate that the aging hippocampus retained

responsiveness to estrogens. However, they also suggest that less Akt is mobilized to the active synaptic zone where it could influence synaptic efficacy.

As stated above, the estrogen-induced increase in pAkt-IR in young rat is similar to that seen previously for ER α -IR and pLIMK-IR in the CA1 stratum radiatum (Adams et al., 2002; Yildirim et al., 2008). Moreover, the estrogen-induced changes in pAkt-IR observed in aged rats is similar to results observed previously for the NR1 and NR2B receptor subunits in aged rats following estrogen administration (Adams et al., 2001c; Adams et al., 2004). In contrast however, the increases in pAkt-IR seen in aged rats are different from the observation for ER α -IR and pLIMK-IR in aged rats. Notably, estrogen failed to increase ER α -IR and pLIMK-IR in aged rats. Thus, synapses in the aging hippocampus, while remaining responsive to estrogen in terms of Akt phosphorylation, show a decline in such E responsiveness that may be due in part to the reduction of ER α , any intermediate co-activating signaling component (Brann et al., 2008), or even available Akt itself, in some, but not all, of the synaptic compartments. In support, ER α -IR in aged rats is not altered in the cytoplasmic regions (e.g., the reserve pool) of the terminals and dendritic spines in response to estrogen (Adams et al., 2002).

Estrogen also plays an important role in neuroprotection via the pAkt pathway

Akt is an estrogen-responsive signaling intermediate (Ivanova et al., 2002; Mannella and Brinton, 2006) that is known to participate in regulating protein translation via the rapamycin sensitive mammalian target of rapamycin (mTOR) kinase pathway (Gingras et al., 2001; Raught et al., 2001). Therefore, one of the proposed mechanisms of the estrogen-dependent increase in spine number may be through Akt activation, which would lead to synthesis of new synaptic proteins such as PSD-95 (Akama and McEwen, 2003; Znamensky et al., 2003).

Additionally, besides the involvement of Akt in protein translation and cell growth, other key functions of Akt are cell survival, anti-apoptosis, and neuroprotection (Franke et al., 1997). Recent studies have highlighted that female sex hormones represent potential neuroprotective agents against damage produced by acute and chronic injuries in the adult brain (Amantea et al., 2005). It is clear that rapid, non-genomic signaling is important for any neuroprotective estrogen effect as well as for any effect on protein translation (Bryant et al., 2006), especially modulation of cell death regulators, including BAD (Bcl2-associated death promoter) (Amantea et al., 2005).

Akt-dependent neuroprotection can be afforded by downstream anti-apoptotic action and BAD phosphorylation (Datta et al., 1997), and Akt kinase also targets one of the anti-apoptotic pathways, namely, the phosphorylation-dependent inhibition of glycogen synthase kinase 3 β (GSK3 β) which has been identified in mediating hippocampal neuronal survival after transient global ischemia (Endo et al., 2006) and suggested in playing in Alzheimer's Disease-related tau phosphorylation (Shi et al., 2008). Activated Akt decreases apoptotic cytochrome c release, and the activation of the Akt pathway blocks apoptosis by phosphorylating the GSK3 β (Zhao et al., 2005a).

The activation of Akt also feeds into processes in which IGF-1 receptors also play a role (Cardona-Gomez et al., 2001). The interaction between estradiol and IGF-1 is also of particular physiological relevance for the regulation of growth, sexual maturation and adult neuroendocrine function (Cardona-Gomez et al., 2001). Both *in vitro* and *in vivo* studies have shown that there is an interaction between ERs and IGF-1R in the promotion of neuronal survival and in the response of neural tissue to injury (Cardona-Gomez et al., 2001). Furthermore, estrogen-induced Akt phosphorylation is an essential mediator of IGF-1 neuroprotective actions (D'Astous et al., 2006). At the same time, IGF-1R is necessary for

the estradiol-induced expression of the anti-apoptotic molecule Bcl-2 (Cardona-Gomez et al., 2001; Honda et al., 2001).

Estrogen, Akt, and synaptic maintenance in the aged brain

Akt is a signal transduction intermediate that promotes protein translation-dependent cell growth and is neuroprotective. Our findings that estrogen administration can stimulate an increase in pAkt-IR which is associated with Akt activity is consistent with previously demonstrated *in vitro* and *in vivo* results (Akama and McEwen, 2003; Znamensky et al., 2003). Our findings also continue to provide a synaptogenesis-promoting and neuroprotective functionality for the dendritic spine-localized ER's which we have demonstrated previously (Milner et al., 2005; Romeo et al., 2005; Waters et al., 2009).

Because estrogen can increase pAkt-IR in aged synapse while other synaptic markers decline or show no changes, it is possible therefore that pAkt-IR in the aged rat brain could serve as an *in vivo* validation screen for functional selective estrogen receptor modulator-based therapeutics (Zhao et al., 2005b) designed to mimic and administered to stimulate synaptic maintenance and neuroprotection in the aging brain. However, it is important to keep in mind that as we have also demonstrated, in the aged hippocampus, estrogen can increase the number of synaptic NMDA receptors (Adams and Morrison, 2003; Adams et al., 2004). Conceivably, this age-dependent increase in estrogen-stimulated NMDA receptors might create an imbalance over diminishing Akt-mediated neuroprotection. This eventual imbalance could result in synaptic hyper-stimulation by excess NMDA receptors and cell death (Balazs, 2006; Hetman and Kharebava, 2006). If we can identify the age at which age-dependent Akt sensitivity to estrogen begins to decline but before age-dependent NMDA receptor hyper-sensitivity begins to emerge, we might be able to characterize a "window of opportunity" to intervene with appropriate treatments against age-related memory decline in menopause (Gibbs, 2000; Gibbs and Gabor, 2003; Sherwin, 2009).

EXPERIMENTAL PROCEDURE

Animals, ovariectomy procedures and estrogen replacement

Eleven young (3-4 month; 225 gm) and twelve aged (23-24 month; 350 gm) female Sprague-Dawley rats from Harlan (Indianapolis, IN) were used in the post-embedding immunogold electron microscopic analyses. These same animals have been used in our previous studies analyzing the effects of aging and E on synapse number (Adams et al., 2001c), synaptic ER α -IR (Adams et al., 2002), synaptic NMDA receptor (Adams et al., 2004) and pLIMK (Yildirim et al., 2008). Rats were housed in a temperature-controlled room (12-h light/dark cycle; lights on at 0700). Food and water were available *ad libitum*. All experiments were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals and with protocols approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine.

Bilateral OVX was performed under isoflurane (2-3%) anesthesia in O₂ using modified previously published surgical guidelines (Eddy, 1986). Seven days later, the rats were reanesthetized with isoflurane and half of the rats from each age group were implanted (s.c.) with a silastic capsule (capsule dimensions: outer diameter 3.18 mm; inner diameter 1.96 mm) filled with the estrogen 17 β -estradiol benzoate (E, 10% in cholesterol), and the other half of the rats were implanted with an implant containing cholesterol only (O). Young animals received an implant that was 1 cm in length and aged animals received an implant that was 2 cm in length to account for differences in body weight (Funabashi et al., 1998; Lauber et al., 1990).

All young rats had regular estrous cycles and aged rats had acyclic estrous cycles (i.e., constant estrus or diestrus), as was demonstrated previously in our laboratory in a different cohort of animals (Adams et al., 2001a). In addition, the effectiveness of the E regimen as compared to vehicle after OVX was determined by examining the uterus from each animal. In young and aged rats administered with vehicle, uteri were very small and atrophied, whereas those rats that had received E had enlarged uteri. We previously used a similar E replacement paradigm in longer-term OVX aged animals and observed a similar uterine response and that the circulating E levels were within a physiological range (Adams et al., 2001b).

Post-embedding electron microscopic immunocytochemistry

Tissue preparation—Animals were anesthetized with 30% chloral hydrate (i.p.) and perfused transcardially with 2% dextran in 0.1 M phosphate buffer (PB) (pH 7.4, 50 ml/min) for 1 min, followed by 4% paraformaldehyde and 0.125% glutaraldehyde in PB for 10–15 min (Adams et al., 2001c; Adams et al., 2002; Adams et al., 2004). The carcasses were examined to confirm complete removal of both ovaries and the presence of the implants. No animal in the present study had any incomplete ovary removal or any obvious pituitary tumors. The brains were removed from the skull and incubated overnight in the fixative at 4°C. Two coronal blocks from the dorsal hippocampus (1 mm thick) were randomly selected from each animal and processed for post-embedding immunogold labeling.

Freeze substitution and low-temperature embedding of the specimens were performed as described previously (Adams et al., 2002; Janssen et al., 2005; van Lookeren Campagne et al., 1991). Hippocampal slices were cryoprotected by immersion in increasing concentrations of glycerol (10%, 20%, and 30% in PB) and were plunged rapidly in liquid propane cooled by liquid nitrogen (−190°C) in a Universal Cryofixation System KF80 (Reichert-Jung, Vienna). The samples were immersed in 1.5% uranyl acetate (for *en bloc* fixation) in anhydrous methanol (−90°C, 24 h) in a cryosubstitution Automatic Freeze-Substitution System unit (Leica, Vienna). The temperature was increased in steps of 4°C/h from −90°C to −45°C. The samples were washed with anhydrous methanol and infiltrated with Lowicryl HM20 resin (EMS, Washington, PA) at −45°C with a progressive increase in the ratio of resin to methanol for 1 h each, followed by pure Lowicryl (overnight). Polymerization was performed with UV light (360 nm) at −45°C for 48 h, followed by 24 h at room temperature.

Anti-pAkt antibody—The anti-pAkt antibody (Cell Signaling Technologies, Beverly, MA), which was used in this study, has been well characterized (Ballou et al., 2000). It is a polyclonal antibody generated in rabbit with a synthetic phospho-peptide (KLH coupled) corresponding to residues surrounding Thr308 of mouse Akt (Alessi et al., 1996). The antibody was purified by protein A and peptide affinity chromatography. Specificity of the antibody was determined using Western blot analyses (Akama and McEwen, 2003), where it reveals a single band at the appropriate molecular weight. The pAkt antibody does not detect non-phosphorylated Akt or Akt phosphorylated at other sites nor does it cross-react with related family members such as PKC or p70 S6 kinase. Our previous studies (Akama and McEwen, 2003; Spencer et al., 2008; Znamensky et al., 2003) have used this antibody.

Immunocytochemical procedure—Sections (75 nm thick) from an area (150–200 μm from the cell bodies) in the stratum radiatum of CA1 were cut using a diamond knife on a Reichert-Jung ultramicrotome and collected on nickel mesh grids. At room temperature, the mesh grids with ultrathin sections were incubated with a saturated solution of NaOH in absolute ethanol (about 10 min) and rinsed in distilled water. The grids then were incubated

in 0.1% sodium borohydride and 50 mM glycine in PB (10 min), followed by Tris-buffered saline (TBS; pH 7.6) containing 2% human serum albumin (HSA) (10 min).

Sections were incubated with primary rabbit polyclonal anti-pAkt antibody (diluted 1:17.5) overnight at room temperature. The next day, sections were washed in TBS, and incubated in secondary gold-tagged (10 nm) goat-anti-rabbit IgG F(ab)₂ antibody (EMS; diluted 1:75) in TBS with 2% HSA and polyethyleneglycol 20,000 (5 mg/ml). The primary anti-pAkt antibody and secondary antibody dilutions for quantitative electron microscopy were determined empirically. Sections were washed in distilled water, dried and then counterstained with 1% uranyl acetate and Reynolds lead citrate. The sections were analyzed on a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan). Images were captured using an Advantage charge-coupled device camera (Advanced Microscopy Techniques, Danvers, MA).

To control for the specificity of the labeling by the anti-pAkt antibody, grids with thin sections from each animal were processed as described above except that the primary antibody was omitted. To control for nonspecific adsorption of the protein-gold complex of the secondary antibody to the tissue, the secondary antibody was replaced with non-conjugated secondary antibody at the same protein concentration. In both controls, only occasional immunogold particles were seen and they were randomly distributed.

Quantitative analysis—Any synaptic complex that contained at least two immunogold particles per post-synaptic profile (i.e., in the synaptic cleft, post-synaptic density, or spine head) was considered positive for pAkt-IR (Adams et al., 2002; Adams et al., 2004). Images were taken at a final magnification of 7,500x. For percent pAkt-IR, at least 150 synapses were collected for each animal and the analyses were performed blind to age and E treatment. All images were captured and further processed by using the Adobe Photoshop 9.0 program. Only minor adjustments of brightness and contrast and sharpening were made, which in no case altered the appearance of the original material.

The immunogold particle density and distribution in various compartments was analyzed using SYNBIN software (Adams et al., 2001c; Adams et al., 2002; Adams et al., 2004) that is based on principles regarding proximity to membranes (Ruud and Blackstad, 1999). As described previously (Yildirim et al., 2008), the position of each immunogold particle was determined relative to the pre- and post-synaptic membranes. The bin sizes are established to accurately reflect immunogold particle distribution and density in different compartments of the synaptic complex (Adams et al., 2002; Adams et al., 2004). Immunogold particle analysis was done on an average of 40 randomly chosen spines per animal at a final magnification of 40,000. Synapses cut obliquely that lacked clear visualization and delineation of classic synaptic structures such as pre- and post-synaptic membranes, a synaptic cleft, and post-synaptic density were excluded from the quantitative analysis.

For the present analysis, 30 nm was chosen for the bin width because it assures high resolution yet comfortably accommodates the theoretical limit of resolution of the immunogold technique (i.e., 25 nm). As shown in Figure 3, eight zones were defined for each synapse (Adams et al., 2001c; Adams et al., 2002; Adams et al., 2004): (1) two post-synaptic bins, the first bin 0-30 nm from the inner leaflet of the post-synaptic membrane [Bin 1] and the second bin 30-60 nm from the post-synaptic membrane [Bin 2]; (2) peri-synaptic bins that were 15 nm lateral to both of the post-synaptic bins [Bin 3]; (3) a cytoplasmic bin that included immunogold particles >60 nm from the post-synaptic membrane [Bin 4]; (4) the synaptic cleft [Bin 5]; (5) two pre-synaptic zones, one extending 0-30 nm from the inner border of the pre-synaptic membrane [Bin 6] and the second extending 30-60 nm from the inner border of the pre-synaptic membrane [Bin 7]; and (6) a

pre-synaptic bin that included immunogold particles >60 nm from the pre-synaptic membrane [Bin 8]. With such a design, immunogold particles in the 0-30 nm PSD bin are unquestionably synaptic in location, whereas all other post-synaptic bins may include particles representing non-synaptic pools of pAkt.

Statistical analysis—Statistical analyses were performed using StatView 5.0 (Abacus Concepts, Inc., Berkeley, CA). The coefficients of variation (CV = standard deviation/mean) and coefficient error (CE) were calculated. Potential group differences in percentage of labeled synapses (Fig. 2) were evaluated by a two-factor (age by treatment) analysis of variance (ANOVA). In figure 4, each bin was analyzed individually with a two-factor (age by treatment) ANOVA. Significance was set at $p < 0.05$. All values are given as means $\pm$ SEM.

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Fig. 1. Representative electron micrographs show the distribution of pAkt-IR (black particles) in the CA1 stratum radiatum of young OVX + estrogen (E) (**A**), young OVX + vehicle (**B**), aged OVX + E (**C**) and aged OVX + vehicle (**D**) rats. t, terminal; sp, dendritic spine. Bar, 100 nm.

Fig. 2.

The overall percentage of pAkt immunoreactive axospinous synapses is significantly decreased in aged OVX rats compared to young OVX rats and significantly increased following E-replacement in both young and aged OVX rats. * $p < 0.02$.

Fig. 3.
Schematic drawing showing the eight bin divisions of a pre- and post-synaptic profile.

Fig 4.

Both age and estrogen altered the subcellular distribution of pAkt immunogold particles in pre- and post-synaptic profiles in the CA1 stratum radiatum region. **A.** In young OVX rats, E administration significantly increased the number of pAkt immunogold particles in all synaptic bins except 6 and 7 (although there was a trends in bin 7, $p = 0.05$). **B.** In aged OVX rats, E administration significantly increased the number of pAkt immunogold particles only in bins 1, 2 and 6. Moreover, E administration to aged OVX animals significantly decreased the number of pAkt immunogold particles in bin 4. * $p < 0.05$

Fig. 5.

Schematic diagram showing the effects of age and estrogen replacement on the distribution of pAkt in synaptic complexes of the female rat stratum radiatum CA1 region. Synaptic complexes from young OVX rats have more pAkt-IR, especially in the post-synaptic region compared to aged OVX rats. Following E treatment, pAkt-IR is increased in synaptic complexes in both young and aged OVX rats, although the stimulus in young rats is more widespread.