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Chronic Estradiol Treatment Improves Brain Homeostasis during Aging in Female Rats

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Aging is associated with a reduction in metabolic function, insulin resistance, increased incidence of neurodegenerative diseases, and memory or cognitive dysfunction. In aging females, loss of gonadal function determines the beginning of the period of reduced metabolic function. Estrogens have neuroprotective effects, but the mechanisms by which they exert these effects remain unclear. The effects of estradiol treatment on the activation of the insulin receptor substrate (IRS)-1 signaling pathway, the interactions between estrogen receptor (ER)-α and IRS-1 and the p85α subunit of phosphatidylinositol-3 kinase, together with the possible effects of estradiol treatment on glucose transporter-3 and -4 levels, were investigated in female rats. The level of expression of each glucose transporter was greater in control and estradiol-treated groups than in the ovariectomized group. Interactions of ERα46-IRS-1, ERα46-p85α, and p85α-IRS-1, as well as IRS-1 phosphorylation, appeared to increase with estradiol treatment. The results indicate that estradiol treatment improves some aspects of neuronal homeostasis that are affected by aging; this may indicate that estradiol has neuroprotective effects in female rats. Additional animal studies are required to clarify the neuroprotective role of estradiol in relation to other important molecules involved in the IRS-1-phosphatidylinositol-3 kinase signaling pathway. (*Endocrinology* 149: 57–72, 2008)

AGING IS ASSOCIATED with a decline in metabolic function, and one of the main illustrations of this metabolic decline is the development of insulin resistance. Although the mechanism underlying the development of insulin resistance with advanced age remains unclear, in the case of females, loss of gonadal function seems to determine the start of this period of metabolic decline. In addition, insulin resistance in aging is associated with metabolic syndrome, which is associated with increased incidences of depression, neurodegenerative diseases, and memory or cognitive dysfunction (1). Therefore, the increased incidences of neurodegenerative diseases in postmenopausal females seem to be clinically associated with aging, loss of gonadal function, and development of insulin resistance.

In the mammalian brain, experimental studies and clinical observations have demonstrated the importance of estrogens in the preservation of brain cognitive functions (2, 3), highlighting its protective effects against neuronal damage (4, 5). However, it remains unclear whether estrogens can improve some aspects of brain function during aging in females and whether this is the mechanism by which it exerts these estrogen-dependent neuroprotective effects. Estrogen-related neuroprotection can be explained not only by a classical action mechanism (6) but also by a nonclassical action mechanism (4, 7), because estrogens receptors (ERs) have been identified in extranuclear locations (cytosolic and plasma membrane) of brain areas known to be affected during neurodegenerative processes (8). At present, two ERs have been identified, termed α (ERα) and β (ERβ). Both are widely, but not uniformly, distributed in the central nervous system (CNS), and advanced age seems to affect the expression and distribution of both. However, only ERα has been implicated in the activation of the phosphatidylinositol-3 kinase (PI3-k) signaling pathway (8–15).

Evidence has suggested that the neuroprotective effects of estrogens may be mediated by the activation of PI3-k signaling pathways (9, 10), which are mediated by ERs. On the other hand, as in the periphery, insulin's action in the brain is mediated by insulin receptor, although at higher concentrations, insulin also binds IGF receptors. Both receptors can be activated, resulting in autophosphorylation and subsequent phosphorylation of several interacting proteins including those of the insulin receptor substrate (IRS) family, of which IRS-1 is the best characterized. IRS-1 induces the activation of downstream pathways such as the regulatory subunit (85-kDa) of PI3-k, which has a pivotal role in the metabolic actions of insulin, including insulin-stimulated glucose transport through the specific translocation of glucose transporter-4 (Glut-4) from the intracellular pool to the plasma membrane, and the MAPK cascades. These two signaling pathways also constitute insulin receptor downstream cascades in the brain. Therefore, it seems possible that ERα can interact with these signaling pathways, mainly with PI3-k and IRS-1, to promote neuroprotective effects in the brain.

Moreover, the positive effects of estradiol could be related to a fundamental anabolic mechanism underlying estrogen

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Abbreviations: CNS, Central nervous system; ER, estrogen receptor; Glut-4, glucose transporter-4; HSD, honestly significant difference; IRS, insulin receptor substrate; PI3-k, phosphatidylinositol-3 kinase.

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action on the brain. This is because estrogen treatment has been shown to increased cerebral metabolic activity (16) and cerebral glucose utilization (17). In this way, the expression, regulation, and activity of brain glucose transporters have an essential role in neuronal homeostasis because glucose is the main energy source for the brain (18, 19). Glut-1 and Glut-3 are widely expressed in the CNS and seem to be responsible for the most glucose uptake and utilization in the brain (20). Glut-4 has also been found in the brain, but its abundance is low and similar to that found in the peripheral tissues (21). However, the presence of Glut-4, insulin-dependent glucose transporter, in the brain could indicate that insulin directly mediates glucose transport in the CNS (21). Several studies have shown that brain glucose transport and utilization are reduced in elderly patients with mild cognitive impairments (22), and investigations into the effects of diabetes have found similar results (23, 24). Taken together, several studies indicate that estrogen increases glucose uptake and utilization (25).

In an experimental model of aging using ovariectomized rats treated with physiological doses of 17β-estradiol, we previously showed that relatively low doses of 17β-estradiol have beneficial effects on glucose homeostasis; however, the estradiol treatment did not prevent memory and learning impairment associated with aging (26). In the present study, we have assessed whether estradiol treatment induced the activation of the IRS-1 signaling pathway and affects the interaction of ERα with IRS-1 and the p85α subunit of PI3-k, and we investigated possible effects on Glut-3 and Glut-4. This study will improve our understanding of whether utilization of estradiol can improve brain functionality during aging. For these studies, we have selected the cortex and diencephalon regions of the brain, because they are the areas in which the functional interdependence of estradiol and PI3-k has been most extensively characterized.

Materials and Methods

Animals

Virgin female Wistar rats (from the Biotery of the Faculty of Medicine, University of Oviedo) weighing 250–280 g (age 8–10 wk) and kept under standard conditions of temperature (23 ± 3 C) and humidity (65 ± 1%) and a regular 12-h light, 12-h dark cycle (0800–2000 h) were used. The animals were fed with a standard diet (Panlab A04), and all of them had free access to water. All experimental manipulations were performed between 0930 and 1230 h. All experimental procedures carried out with animals were approved by a local veterinary committee from the University of Oviedo vivarium, and subsequent handling strictly followed the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Experimental design

Rats were ovariectomized through a midline incision under light anesthesia by inhalation of halothane. Ovariectomized rats were separated randomly into three groups: ovariectomized animals (OVX), ovariectomized animals treated with 17β-estradiol (E), and sham surgery animals (intact) (C) and housed individually throughout the experiment.

After surgery, all rats began the experimental treatment exactly 1 wk after ovariectomy to ensure a uniform time of estrogen depletion before replacement and to recover from surgery stress. After this, the rats were implanted sc in the posterior neck with 90-d-release 17β-estradiol pellets (25 μg/pellet; Innovative Research of America, Sarasota, FL) or placebos containing no estradiol. Every 90 d, the pellets were replaced. This dosing regimen has resulted in physiological levels of plasma estradiol and has been shown to be neuroprotective in rats (27).

Groups (OVX, E, and C) were divided randomly into four subgroups (seven animals per subgroup): 6, 12, 18, and 24 (according to the month of the experimental period on which the animals were killed). Therefore, the animals were killed when they were 8, 14, 20, and 26 months old approximately. Moreover, 14 animals (seven OVX and seven C) were killed 1 wk after ovariectomy (age 9–11 wk). Therefore, the animals included in this group did not receive any treatment. These animals are considered as OVX-0 month and C-0 month groups.

The stage of the estrous cycle in intact rats was determined by daily examination of fresh vaginal smears. The intact animals in diestrous phase were selected for the 0 and 6 subgroups. None of the intact rats since month 12 of the experiment showed repetitive estrous cycles; instead, 87.26% of them showed persistent diestrous phase.

Euglycemic insulin clamp

Clamp experiments were performed by the previously described procedure (28), and the results were published recently (26). After the clamp study, blood samples for the determination of 17β-estradiol plasma concentrations were collected from the jugular vein into heparinized tubes and centrifuged at 3000 rpm duforring 20 min at 4 C, and plasma was immediately drawn off and stored frozen at −20 C until assayed. Plasma 17β-estradiol was measured by RIA using Immuchen kits of cover tubes (ICN Biomedicals Inc., Costa Mesa, CA). The assay sensitivity was 10 pg/ml, and the intraassay coefficient of variation was 9.45%. All samples were measured on the same day. The sample was assayed in triplicate.

Crude extract preparation, immunoprecipitation, and Western blot analysis

After clamp experiments, the brains were quickly removed. The cortex and diencephalon were dissected and homogenized in lysis buffer [50 mM-Tris HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40 (Roche Diagnostics, Indianapolis, IN), 0.05% sodium deoxycholate, sodium orthovanadate, 5 mM EDTA, 10% glycerol] at 4 C. The extracts were centrifuged at 21,000 × *g* at 4 C for 10 min, and the protein content of the supernatant was determined by the Bradford dye-binding method (29).

First, protein were resolved using SDS-PAGE [10% Tris (hydroxymethyl) aminomethane (Tris)-polyarilamide; Bio-Rad, Barcelona, Spain]) and electrotransferred from the gel to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Little Chalfont, UK) as described by Towbin *et al.* (30). Nonspecific protein binding to the nitrocellulose membranes was reduced by preincubating the filter in blocking buffer [Tris-NaCl-Tween 20 (TNT), 7% BSA] and incubated overnight with the primary antibodies. The antibodies against Glut-3 (sc-7582, diluted 1:1000), Glut-4 (sc-7938, diluted 1:1000), and ERα (sc-542, diluted 1:2000) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Alternatively, according to Marin *et al.* (31), a monoclonal antibody directed to ERα (product no. SRA-1010; Stressgen Biotechnologies, Victoria, Canada) was also used to verify the results obtained with the other anti-ERα antibody. After incubation with the primary antibody, the membranes were washed and incubated with an antigoat antibody coupled to horseradish peroxidase (sc-2768 from Santa Cruz, diluted 1:15,000) or an antirabbit antibody coupled to horseradish peroxidase (sc-2004 from Santa Cruz, diluted 1:15,000). Finally, all membranes were stripped and probed with a monoclonal anti-β-actin antibody (sc-1615 from Santa Cruz, diluted 1:2500). Immunoreactive bands were detected using an enhanced chemiluminescence system (ECL; Amersham). Films were analyzed using a digital scanner (Nikon AX-110) and NIH Image 1.57 software. The density of each band was normalized to its respective loading control (β-actin) and represented as percentage of control values (intact rats of 0 month, group C0). To minimize interassay variations, samples from all animal groups, in each experiment, were processed in parallel.

On the other hand, aliquots containing 500 μg protein from crude homogenate were subject to immunoprecipitation using polyclonal antibodies against IRS-1 (sc-559; Santa Cruz) and the p85α subunit of PI3-k (sc-423; Santa Cruz). Parallel immunoprecipitations were performed using a nonimmune rabbit serum to verify the specificity of the bands

detected by Western blotting. The immune complexes were adsorbed and precipitated using protein G-agarose beads (sc-2002; Santa Cruz) overnight at 4 C in a rocking platform and were washed several times with lysis buffer, suspended in protein loading buffer [250 mM Tris-HCl (pH 6.8), 8% SDS, 8 mM EDTA, 35% glycerol, 2.5% β-mercaptoethanol, bromophenol blue] and denatured in a boiling water bath for 5 min. The immunoprecipitates were resolved by 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Western blotting was performed as described above using an antibody against ERα (sc-542; Santa Cruz; diluted 1:2000), against IRS-1 (sc-559; Santa Cruz), and against the p85 subunit of PI3-k (sc-423; Santa Cruz).

Statistical analysis

Data are expressed as mean ± SEM. Previously, we evaluated the Gaussian distribution of each variable. After this, data were statistically analyzed using an ANOVA design followed by between-group comparisons using the Tukey honestly significant difference (HSD) test. The data for month 0 were analyzed by unpaired Student's *t* test. $P \leq 0.05$ was considered as significant. Statistical analysis was performed using SPSS version 14.0.1 for Windows.

Results

The plasma 17β-estradiol values obtained during the study are shown in Fig. 1. The plasma level of estradiol was significantly higher in the estradiol (E) and control (C) groups than in the ovariectomized (OVX) group at all times. In addition, plasma levels of 17β-estradiol were significantly higher in the E group than in the C group at 18 and 24 months. In the C group animals, plasma levels of 17β-estradiol increased significantly at 6 months and then decreased significantly at 24 months. In the E group, the estradiol level did not change significantly during the study. A significant decrease in the estradiol level was found in the OVX group during the first 6 months.

The effects of aging on Glut-3 and Glut-4 levels in the cortex and diencephalon of the brain were determined. In the cortex of intact rats, a significant increase in the level of Glut-3 was noted over the first 12 months; the Glut-3 level then decreased significantly from 12 months until the end of the study (Fig. 2A). A similar pattern was observed in the OVX group, whereas in the E group, the Glut-3 level increased significantly for the first 18 months and then decreased significantly until the end of the study.

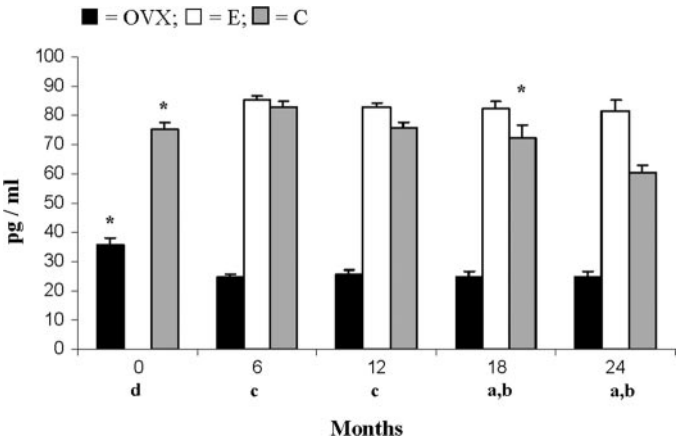


FIG. 1. Levels of 17β-estradiol of intact (C) ($F_{(4,34)} = 22.23$; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)} = 33.37$; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)} = 2.54$; $P = 0.13$) at 0 ($t_{(12,0.05)} = 5.63$; $P \leq 0.05$), 6 ($F_{(2,20)} = 522.3$; $P < 0.0001$), 12 ($F_{(2,20)} = 740.3$; $P < 0.0001$), 18 ($F_{(2,20)} = 150.0$; $P < 0.0001$), and 24 ($F_{(2,20)} = 2050.9$; $P < 0.0001$) months of age. Values are means ± SEM (n = 7). Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E; c, OVX *vs.* C, E; d, OVX *vs.* C.

The amount of Glut-3 in the cortex was significantly lower in the OVX group than in the other groups. Interestingly, for the first 12 months, the Glut-3 level was higher in the C group than in the E group, whereas at 18 and 24 months, the Glut-3 level was higher in the E group than in the C group, although the differences were not statistically significant.

In the C group, the pattern of Glut-3 levels in the diencephalon was similar to that found in the cortex (Fig. 2B). In the OVX group, a significant increase was noted over the first 6 months, followed by a significant decrease that lasted until the end of the study; a similar pattern was observed in the E group.

In the diencephalon, the Glut-3 level was always significantly higher in the C group than in the other groups. At 6 months, the Glut-3 level was significantly higher in the OVX group than in the E group; however, the Glut-3 level was significantly higher in the E group than in the OVX group at all other time points.

The level of Glut-4 in the two areas of the brain studied (Fig. 3, A and B) showed similar patterns in all groups; there was a significant increase over the first 12 months, followed by a significant decrease until the end of the study. However, when the differences between the groups at different times were examined, it was found that the Glut-4 level was lowest in the cortex of the E group at 6 and 12 months, whereas it was lowest in the OVX group at 18 and 24 months. Interestingly, whereas the Glut-4 level was significantly higher in the C group than in the E group at 6 and 12 months, it was significantly higher in the E group than in the C group at 18 and 24 months.

In the diencephalon, the differences in the level of Glut-4 between the groups were similar at 6, 18, and 24 months; the Glut-4 level was significantly higher in the C group than in the other groups and significantly lower in the OVX group than in the other groups (Fig. 3B). However, the Glut-4 level was significantly higher in the E group than in both the other groups at 12 months.

Immunoprecipitation studies were conducted to assess the possible interaction between ERα and IRS-1. In the C group, the pattern of the interaction between IRS-1 and both isoforms of ERα in the cortex was similar (Fig. 4A); there was a significant increase for the first 12 months, followed by a significant decrease toward the end of the study. The results differed in the OVX group; the interactions decreased significantly from the start to the end of the study. In the E group, a different pattern was found for the interaction between IRS-1 and both isoforms of ERα. Although the interaction between ERα67 and IRS-1 decreased significantly from the start to the end of the study, the opposite was observed with respect to the ERα46-IRS-1 interaction. In the cortex, both interactions (ERα67-IRS-1 and ERα46-IRS-1) were significantly greater in the C group than in the OVX and E groups. The ERα67-IRS-1 interaction was significantly greater in the OVX group than in the E group, as was the ERα46-IRS-1 interaction, at 6 and 12

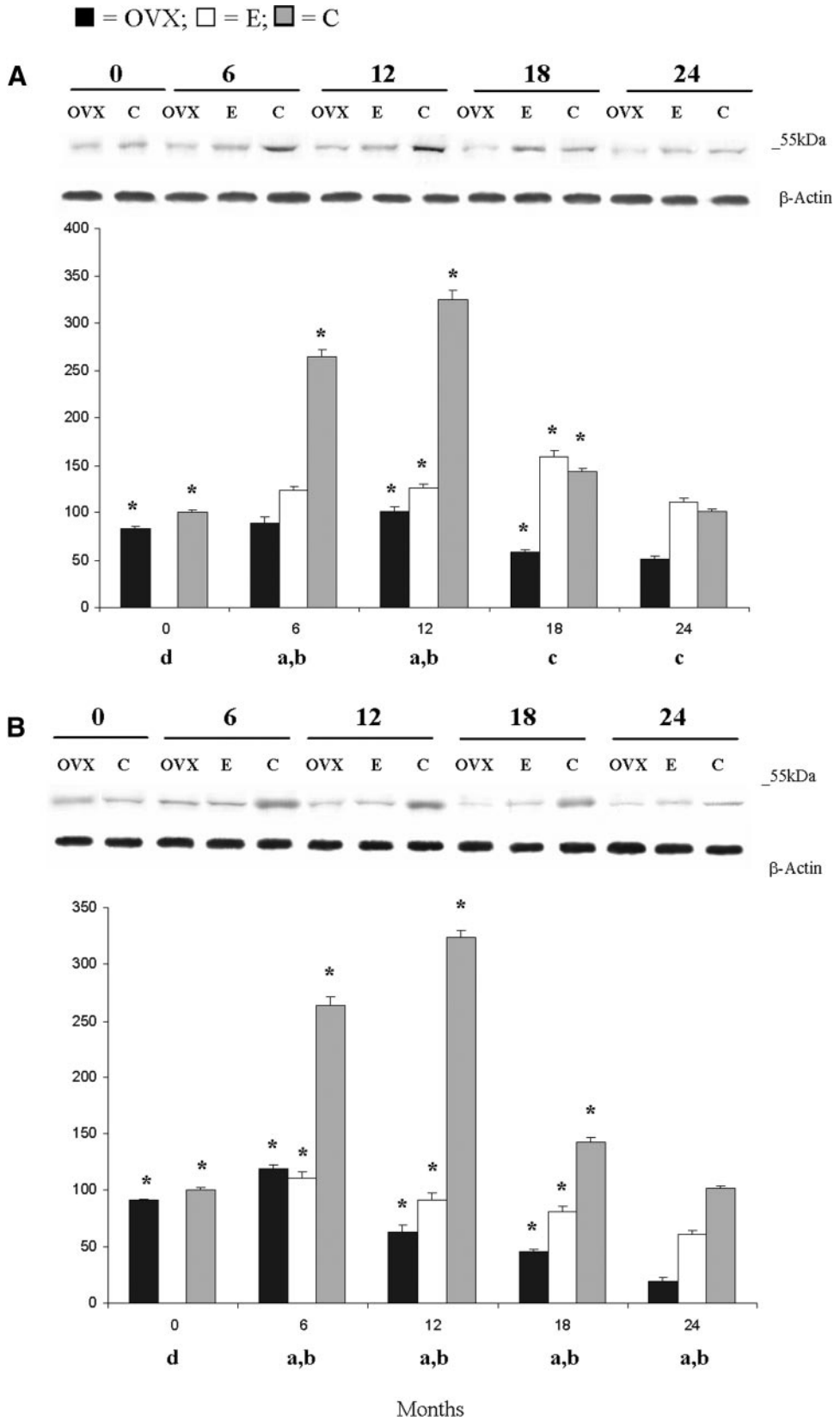


FIG. 2. Glut-3 in cortex (panel A) from intact (C) ($F_{(4,34)} = 8140.2; P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)} = 669.1$; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)} = 1800.6; P < 0.0001$) at 0 ($t_{(12,0.05)} = 5.83$; $P \leq 0.05$), 6 ($F_{(2,20)} = 21,447.3$; $P < 0.0001$), 12 ($F_{(2,20)} = 26,439.3; P < 0.0001$), 18 ($F_{(2,20)} = 4715.5$; $P < 0.0001$), and 24 ($F_{(2,20)} = 9903.2; P < 0.0001$) months of age and in diencephalon (panel B) from intact (C) ($F_{(4,34)} = 9655.4$; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)} = 4843.5$; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)} = 1843.2; P < 0.0001$) at 0 ($t_{(12,0.05)} = 3.86$; $P \leq 0.05$), 6 ($F_{(2,20)} = 22,627.2$; $P < 0.0001$), 12 ($F_{(2,20)} = 29,839.0; P < 0.0001$), 18 ($F_{(2,20)} = 15,537.2$; $P < 0.0001$), and 24 ($F_{(2,20)} = 476.6; P < 0.0001$) months of age. The histogram shows the densitometric analysis of the Western blots. Values are means ± SEM (n = 7) and represented as the percentage of control values (rats of 0 month from group C). Only significant differences are shown. Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E; c, OVX *vs.* C, E; d, OVX *vs.* C.

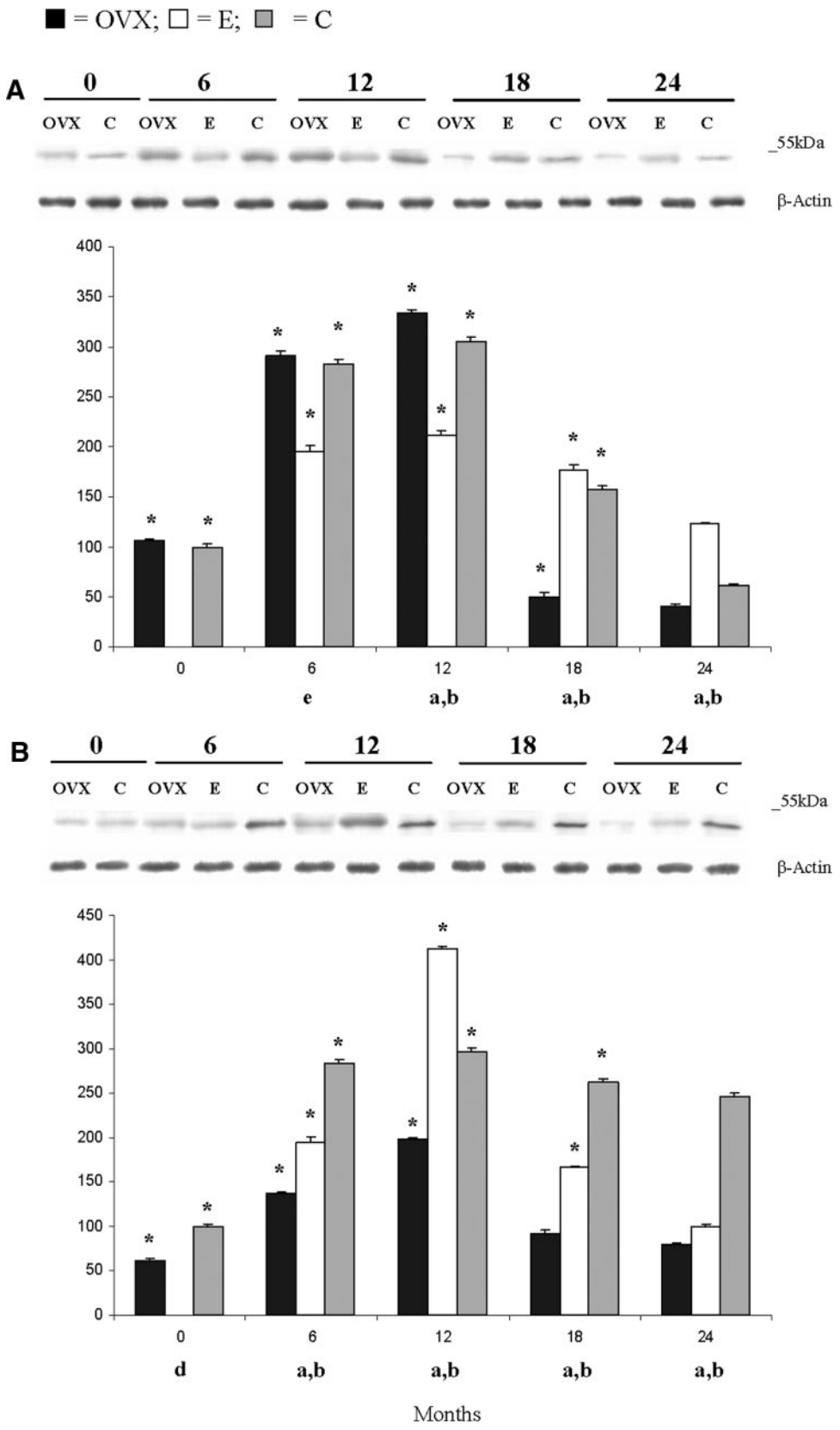
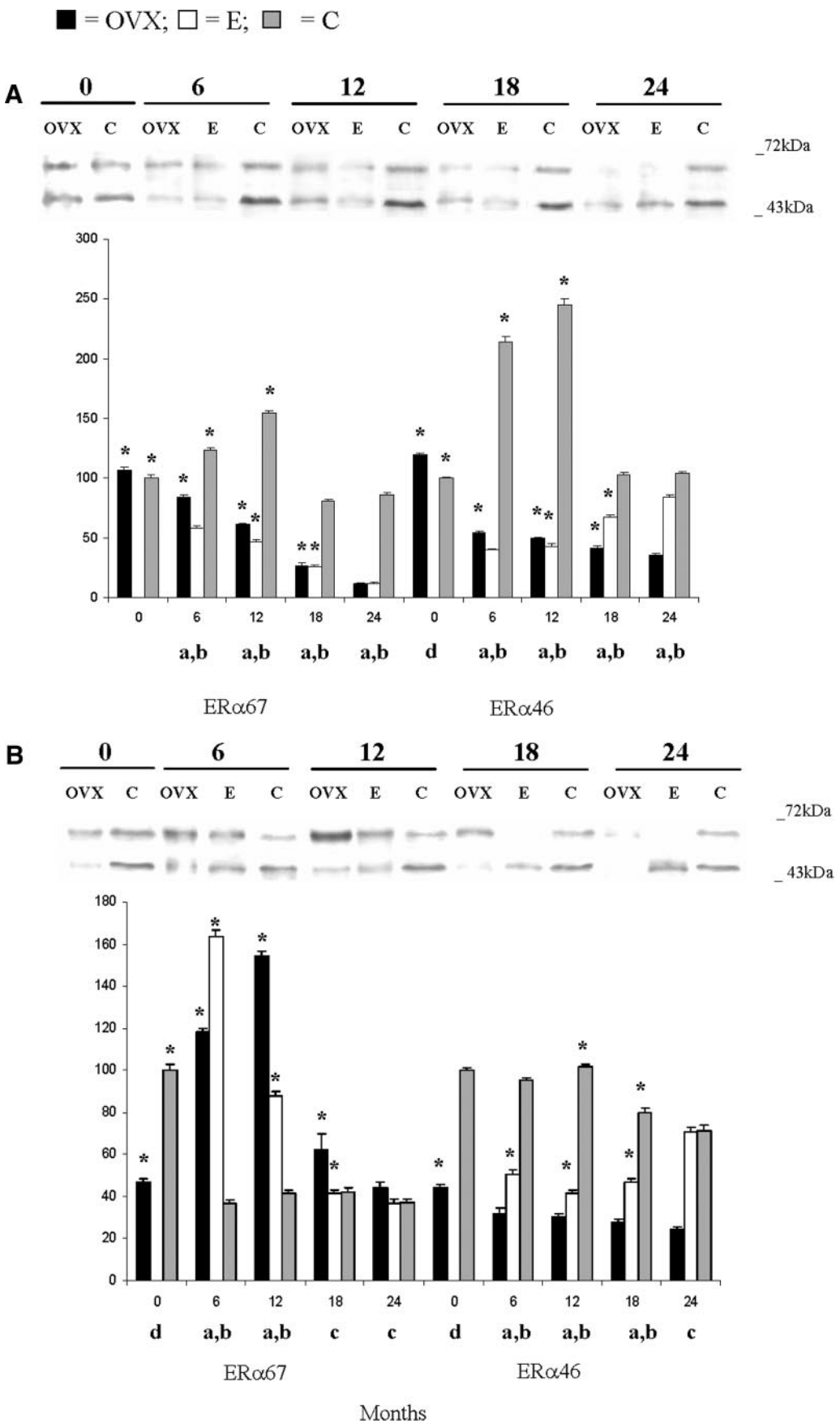


FIG. 3. Glut-4 in cortex (panel A) from intact (C) ($F_{(4,34)} = 638.8$; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)} = 10{,}792.6$; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)} = 403.6$; $P < 0.0001$) at 0 ($t_{(12,0.05)} = 1.07$; P = not significant), 6 ($F_{(2,20)} = 5632.4$; $P < 0.0001$), 12 ($F_{(2,20)} = 143.3$; $P < 0.0001$), 18 ($F_{(2,20)} = 1056.5$; $P < 0.0001$), and 24 ($F_{(2,20)} = 502.4$; $P < 0.0001$) months of age and in diencephalon (panel B) from intact (C) ($F_{(4,34)} = 1354.2$; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)} = 1038.1$; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)} = 38{,}469.8$; $P < 0.0001$) at 0 ($t_{(12,0.05)} = 9.98$; $P \leq 0.05$), 6 ($F_{(2,20)} = 877.7$; $P < 0.0001$), 12 ($F_{(2,20)} = 6351.7$; $P < 0.0001$), 18 ($F_{(2,20)} = 3391.8$; $P < 0.0001$), and 24 ($F_{(2,20)} = 3551.0$; $P < 0.0001$) months of age. The histogram shows the densitometric analysis of the Western blots. Values are means ± SEM (n = 7) and represented as the percentage of control values (rats of 0 month from group C). Only significant differences are shown. Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E; e, E *vs.* OVX, C.



months; however, at 18 and 24 months, the ERα46-IRS-1 interaction was significantly greater in the E group than in the OVX group.

In the diencephalon, the ERα67-IRS-1 interaction decreased significantly from the start of the study to the 6-months time point in the C group, and then no further significant changes were observed to the end of the study (Fig. 4B). In the OVX group, there was a significant increase in the ERα67-IRS-1 interaction over the first 12 months, followed by a significant decrease toward the end of the study. However, in the E group, the ERα67-IRS-1 interaction decreased significantly from 6 months to the end of the study. In contrast, the pattern for the ERα46-IRS-1 interaction showed no significant changes in the C group for the first 12 months, and then there was a significant decrease to 24 months. However, there was a significant decrease in the ERα46-IRS-1 interaction from the start to the end of the study in the OVX group; in the E group, there was a significant decrease in the ERα46-IRS-1 interaction from 6–12 months, followed by a significant increase from 12–24 months.

The ERα67-IRS-1 interaction was significantly higher in the C group than in the others at 0 months, in the E group than in the others at 6 months, and in the OVX group than in the others at 12, 18, and 24 months. However, the ERα46-IRS-1 interaction was always significantly higher in the C group than in the other two groups and always significantly lower in the OVX group than in the other groups (Fig. 4B).

Changes in the tyrosine phosphorylation of IRS-1 during the aging process were also assessed. In the cortex of the C and OVX groups, the level of IRS-1 phosphorylation increased significantly for the first 6 months, decreased significantly from 6–12 months, increased significantly from 12–18 months, and then remained stable until the end of the study (Fig. 5A). In contrast, phosphorylation of IRS-1 in the E group increased significantly until the end of the study. Phosphorylation of IRS-1 was significantly greater in the E group than in the other groups at 12, 18, and 24 months and significantly higher in the C group than in the OVX group at 0, 18, and 24 months.

In the diencephalon, the patterns of tyrosine phosphorylation of IRS-1 were similar to those observed in the cortex in the C and E groups (Fig. 5B). In contrast, in the OVX group, phosphorylation of IRS-1 decreased significantly from the start to the end of the study. In the diencephalon, the phosphorylation of IRS-1 was significantly greater in the C group than in the other groups, except at 12 months. In addition, phosphorylation of IRS-1 was significantly higher in the E group than in the OVX group, except at 6 months.

The interaction between ERα and the p85α subunit of PI3-k was also studied. Interestingly, an ERα67-p85α interaction was detected in the cortex and diencephalon in the C group but not in the OVX and E groups (Fig. 6, A and B). In the cortex of the C group, the ERα67-p85α and ERα46-p85α interactions showed a similar pattern; there was a significant increase until 6 months and a significant decrease from 6–12 months, and then the interactions remained stable until the end of the study (Fig. 6A). In the OVX group, the ERα46-p85α interaction decreased significantly over the first 6 months, followed by a significant increase from 6 months to the end of the study. In the cortex of rats in the E group, the ERα46-p85α interaction increased significantly from 6 months to the end of the study. The ERα46-p85α interaction was always significantly greater in the C group than in the other groups and was always significantly lower in the OVX group than in the other groups, except at the beginning of the study.

In the diencephalon, both the ERα67-p85α and the ERα46-p85α interaction showed similar patterns in the C group: there was a significant increase over the first 12 months, followed by a significant decrease toward the end of the study (Fig. 6B). In the OVX group, there was a significant decrease in the ERα46-p85α interaction until the 24-month time point, whereas the opposite was found in the E group. The ERα46-p85α interaction was always significantly greater in the C group than in the other groups and was always significantly lower in the OVX group than in the other groups, except at 6 months.

Changes in the level of phosphorylation of IRS-1 indicate that activation of the intracellular IRS-1 pathways is involved in aging. Therefore, the interaction between the p85α subunit of PI3-k and IRS-1 was studied. In the cortex, this interaction increased significantly for the first 6 months and then decreased significantly from 6–24 months in the C group (Fig. 7A). A similar pattern was observed in the OVX group. However, in the E group, IRS-1 phosphorylation levels decreased significantly from 6–12 months and then increased significantly from 12 months to the end of the study. At 6 and 24 months, the p85α-IRS-1 interaction was significantly greater in the E

FIG. 4. Association between ERα and IRS-1 in cortex (panel A) from intact (C) (ERα67: $F_{(4,34)} = 1078.0$; $P < 0.0001$; ERα46: $F_{(4,34)} = 5545.0$; $P < 0.0001$), ovariectomized (OVX) (ERα67: $F_{(4,34)} = 7656.6$; $P < 0.0001$; ERα46: $F_{(4,34)} = 100{,}697.2$; $P < 0.0001$), and estradiol-treated (E) rats (ERα67: $F_{(4,34)} = 401.6$; $P < 0.0001$; ERα46: $F_{(4,34)} = 482.7$; $P < 0.0001$) at 0 (ERα67: $t_{(12,0.05)} = 0.74$; P = nonsignificant; ERα46: $t_{(12,0.05)} = 5.69$; $P \leq 0.05$), 6 (ERα67: $F_{(2,20)} = 801.3$; $P < 0.0001$; ERα46: $F_{(2,20)} = 26{,}846.5$; $P < 0.0001$), 12 (ERα67: $F_{(2,20)} = 1209.0$; $P < 0.0001$; ERα46: $F_{(2,20)} = 28{,}380.1$; $P < 0.0001$), 18 (ERα67: $F_{(2,20)} = 2458.1$; $P < 0.0001$; ERα46: $F_{(2,20)} = 1042.6$; $P < 0.0001$), and 24 (ERα67: $F_{(2,20)} = 1802.6$; $P < 0.0001$; ERα46: $F_{(2,20)} = 761.9$; $P < 0.0001$) months of age and in diencephalon (panel B) from intact (C) (ERα67: $F_{(4,34)} = 1079.9$; $P < 0.0001$; ERα46: $F_{(4,34)} = 1626.7$; $P < 0.0001$), ovariectomized (OVX) (ERα67: $F_{(4,34)} = 72{,}360.9$; $P < 0.0001$; ERα46: $F_{(4,34)} = 8399.9$; $P < 0.0001$), and estradiol-treated (E) rats (ERα67: $F_{(4,34)} = 5905.3$; $P < 0.0001$; ERα46: $F_{(4,34)} = 361.5$; $P < 0.0001$) at 0 (ERα67: $t_{(12,0.05)} = 6.22$; $P \leq 0.05$; ERα46: $t_{(12,0.05)} = 5.72$; $P \leq 0.05$), 6 (ERα67: $F_{(2,20)} = 7705.1$; $P < 0.0001$; ERα46: $F_{(2,20)} = 11{,}575.5$; $P < 0.0001$), 12 (ERα67: $F_{(2,20)} = 4037.3$; $P < 0.0001$; ERα46: $F_{(2,20)} = 19{,}505.2$; $P < 0.0001$), 18 (ERα67: $F_{(2,20)} = 171.3$; $P < 0.0001$; ERα46: $F_{(2,20)} = 688.7$; $P < 0.0001$), and 24 (ERα67: $F_{(2,20)} = 36.17$; $P < 0.0001$; ERα46: $F_{(2,20)} = 9033.8$; $P < 0.0001$) months of age. IRS-1 was immunoprecipitated from total lysates as described in *Materials and Methods*. The immunocomplexes were separated using SDS-PAGE and probed with an antibody against ERα. The histogram shows the densitometric analysis of the Western blots. Values are means ± SEM (n = 7), and represented as the percentage of control values (rats of 0 month from group C). Only significant differences are shown. Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E; c, OVX *vs.* C, E; d, OVX *vs.* C.

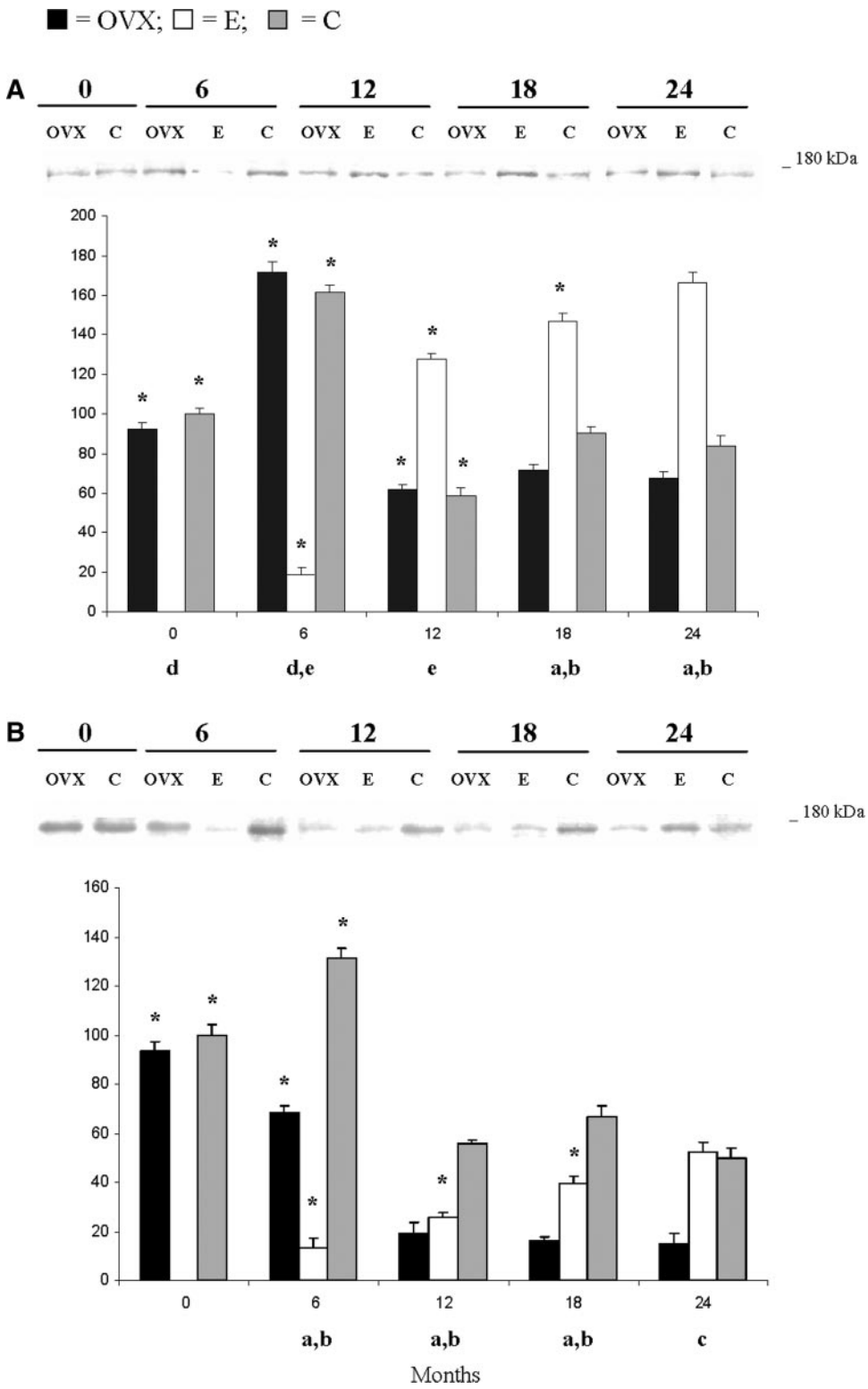


FIG. 5. IRS-1 tyrosine phosphorylation in cortex (panel A) from intact (C) ($F_{(4,34)} = 1510.4$; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)} = 326.2$; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)} = 1522.5$; $P < 0.0001$) at 0 ($t_{(12,0.05)} = 7.39$; $P \leq 0.05$), 6 ($F_{(2,20)} = 720.2$; $P < 0.0001$), 12 ($F_{(2,20)} = 447.5$; $P < 0.0001$), 18 ($F_{(2,20)} = 3296.6$; $P < 0.0001$), and 24 ($F_{(2,20)} = 3084.6$; $P < 0.0001$) months of age and in diencephalon (panel B) from intact (C) ($F_{(4,34)} = 595.2$; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)} = 12{,}146.5$; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)} = 749.2$; $P < 0.0001$) at 0 ($t_{(12,0.05)} = 0.65$; P = nonsignificant), 6 ($F_{(2,20)} = 1806.1$; $P < 0.0001$), 12 ($F_{(2,20)} = 623.0$; $P < 0.0001$), 18 ($F_{(2,20)} = 581.7$; $P < 0.0001$), and 24 ($F_{(2,20)} = 5094.3$; $P < 0.0001$) months of age. IRS-1 was immunoprecipitated from total lysates as described in *Materials and Methods*. The immunocomplexes were separated using SDS-PAGE and probed with an antibody against phosphotyrosine. The histogram shows the densitometric analysis of the Western blots. Values are means ± SEM (n = 7), and represented as the percentage of control values (rats of 0 month from group C). Only significant differences are shown. Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E; c, OVX *vs.* C, E; d, OVX *vs.* C; e, E *vs.* OVX, C.

group than in the others, whereas at 12 and 18 months, it was significantly greater in the C group than in the others.

In the diencephalon, the p85α-IRS-1 interaction decreased significantly in the C group throughout the experiment (Fig. 7B). In the OVX group, this interaction decreased significantly from 6 months to the end of the study, whereas in the E group, it decreased significantly until 12 months and then increased significantly from 12–24 months. At 6 and 24 months, the p85α-IRS-1 interaction in the diencephalon was significantly greater in the E group than in the others, whereas at 12 and 18 months, it was significantly greater in the C group than in the others.

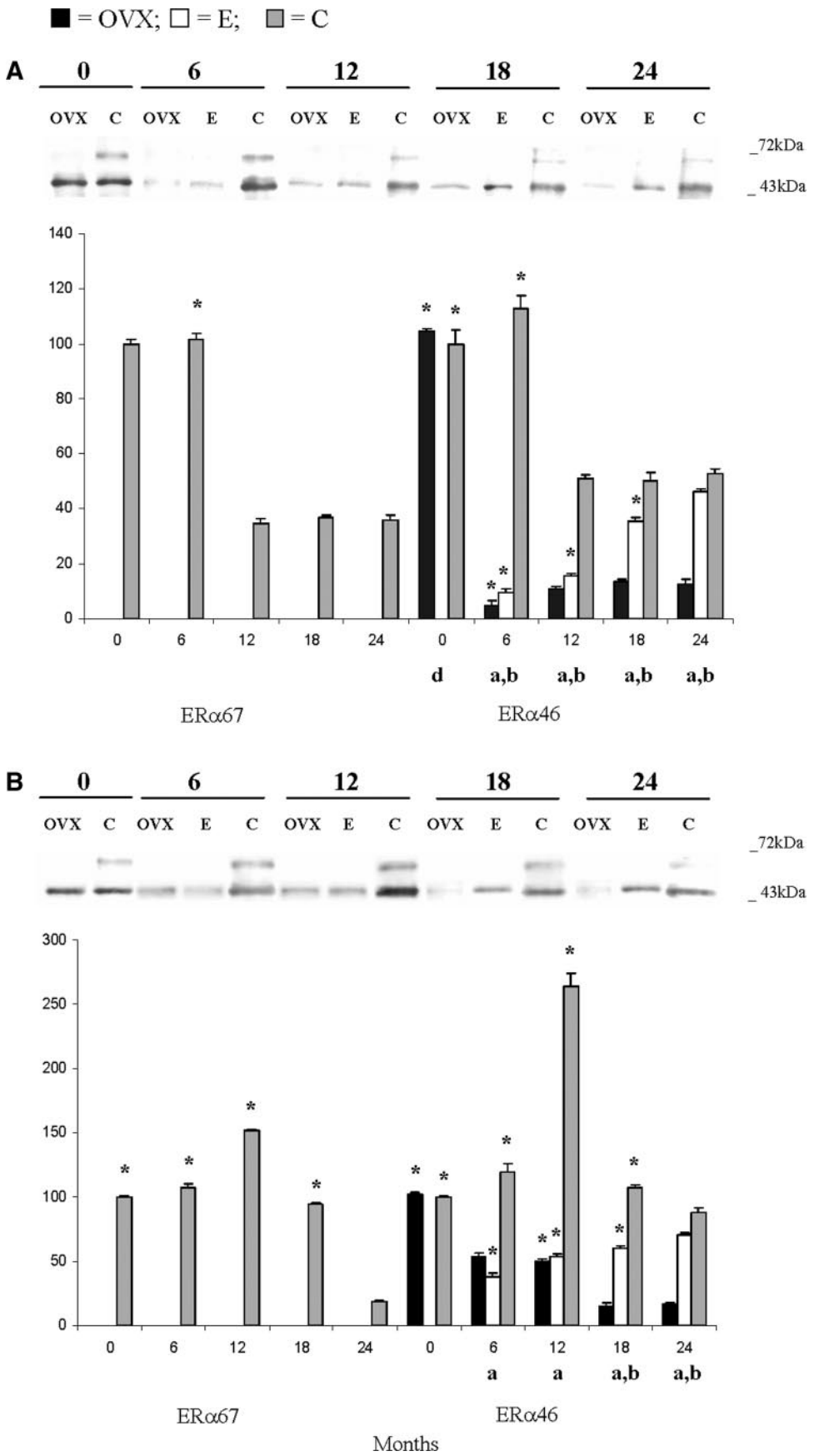


FIG. 6. Association between ERα and p85 subunit of PI3-k in cortex (panel A) from intact (C) (ERα67: $F_{(4,34)} = 1565.3$; $P < 0.0001$; ERα46: $F_{(4,34)} = 19{,}871.7$; $P < 0.0001$), ovariectomized (OVX) (ERα46: $F_{(4,34)} = 26{,}940.6$; $P < 0.0001$), and estradiol-treated (E) rats (ERα46: $F_{(4,34)} = 4537.9$; $P < 0.0001$) at 0 (ERα46: $t_{(12,0.05)} = 3.62$; $P \leq 0.05$), 6 (ERα46: $F_{(2,20)} = 80{,}230.9$; $P < 0.0001$), 12 (ERα46: $F_{(2,20)} = 19{,}215.8$; $P < 0.0001$), 18 (ERα46: $F_{(2,20)} = 35{,}475.1$; $P < 0.0001$), and 24 (ERα46: $F_{(2,20)} = 3978.5$; $P < 0.0001$) months of age and in diencephalon (panel B) from intact (C) (ERα67: $F_{(4,34)} = 2469.2$; $P < 0.0001$; ERα46: $F_{(4,34)} = 8090.2$; $P < 0.0001$), ovariectomized (OVX) (ERα46: $F_{(4,34)} = 7242.1$; $P < 0.0001$), and estradiol-treated (E) rats (ERα46: $F_{(4,34)} = 498.8$; $P < 0.0001$) at 0 (ERα46: $t_{(12,0.05)} = 0.64$; P = nonsignificant), 6 (ERα46: $F_{(2,20)} = 6697.5$; $P < 0.0001$), 12 (ERα46: $F_{(2,20)} = 25{,}695.6$; $P < 0.0001$), 18 (ERα46: $F_{(2,20)} = 4915.8$; $P < 0.0001$), and 24 (ERα46: $F_{(2,20)} = 5003.4$; $P < 0.0001$) months of age. p85 subunit of PI3-k was immunoprecipitated from total lysates as described in *Materials and Methods*. The immunocomplexes were separated using SDS-PAGE and probed with an antibody against ERα. The histogram shows the densitometric analysis of the Western blots. Values are means ± SEM (n = 7) and represented as the percentage of control values (rats of 0 month from group C). Only significant differences are shown. Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E; d, OVX *vs.* C.

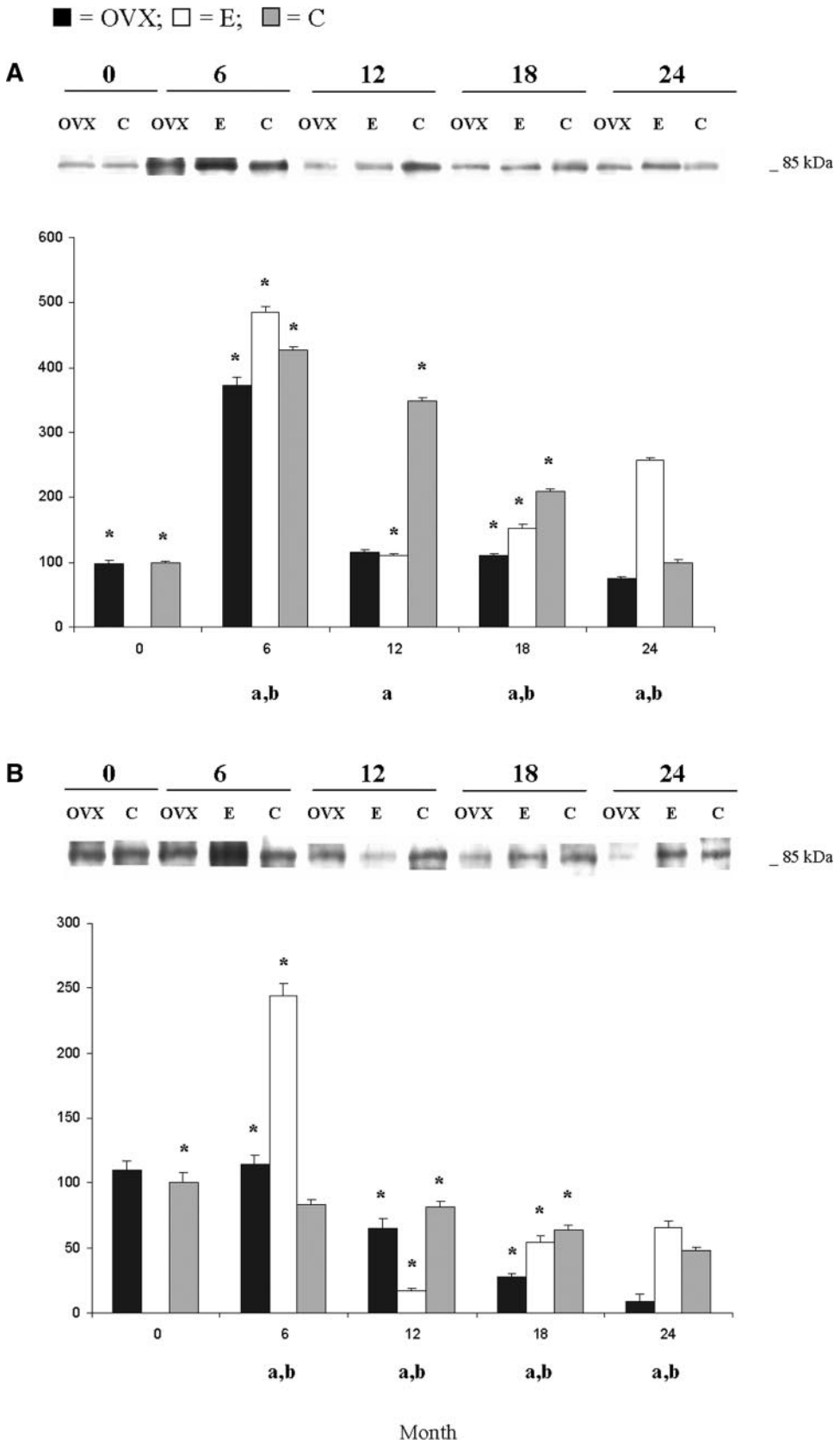


FIG. 7. Association between p85 subunit of PI3-k and IRS-1 in cortex (panel A) from intact (C) ($F_{(4,34)}$ = 2809.7; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)}$ = 11,601.7; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)}$= 71,971.7; $P < 0.0001$) at 0 ($t_{(12,0.05)}$ = 0.53; P = nonsignificant), 6 ($F_{(2,20)}$ = 5312.4; $P < 0.0001$), 12 ($F_{(2,20)}$ = 8209.9; $P < 0.0001$), 18 ($F_{(2,20)}$ = 2241.9; $P < 0.0001$), and 24 ($F_{(2,20)}$ = 4502.0; $P < 0.0001$) months of age and in diencephalon (panel B) from intact (C) ($F_{(4,34)}$ = 650.9; $P < 0.0001$), ovariectomized (OVX) ($F_{(4,34)}$ = 4523.8; $P < 0.0001$), and estradiol-treated (E) rats ($F_{(4,34)}$ = 5671.3; $P < 0.0001$) at 0 ($t_{(12,0.05)}$ = 0.44; P = nonsignificant), 6 ($F_{(2,20)}$ = 3846.2; $P < 0.0001$), 12 ($F_{(2,20)}$ = 1931.7; $P < 0.0001$), 18 ($F_{(2,20)}$ = 405.8; $P < 0.0001$), and 24 ($F_{(2,20)}$ = 1682.2; $P < 0.0001$) months of age. IRS-1 was immunoprecipitated from total lysates as described in *Materials and Methods*. The immunocomplexes were separated using SDS-PAGE and probed with an antibody against phosphotyrosine. The histogram shows the densitometric analysis of the Western blots. Values are means ± SEM (n = 7) and represented as the percentage of control values (rats of 0 month from group C). Only significant differences are shown. Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E.

Finally, the estrogen receptor levels in total crude extracts from the cortex and diencephalon were studied using an MC-20 antibody, as previously described (31–34). This technique detects amino acids 580–599 of the ligand-binding domain of ERα but does not recognize ERβ. Two immunoreactive bands of approximately 67 and 46 kDa were identified in both regions of the brain. It was found that aging and/or estradiol treatment can change ERα expression levels. In the cortex, ERα67 increased significantly for the first 6 months and then decreased significantly from 6–24 months in the C group, whereas ERα46 increased significantly for 6 months, decreased significantly from 6–12 months, and then remained stable until the end of the study (Fig. 8A). The pattern was different in the OVX group; both ERα isoforms decreased significantly throughout the experiment. In the E group, both isoforms showed a significant decrease from 6–12 months, followed by a significant increase to the end of the study. Both ERα isoforms were always significantly greater in the C group than in the other groups and significantly lower in the OVX group than in the other groups, except for ERα67 at 12 months.

In the diencephalon region, ERα67 increased significantly in the C group until the 12-month time point and then decreased significantly to the end of the study (Fig. 8B). However, in the OVX and E groups, ERα67 decreased significantly from the start to the end of the study. In the C group, no significant changes were observed in ERα46 for the first 18 months, after which there was a significant decrease to 24 months, whereas there was a significant decrease in ERα46 from the start to the end of the study in the other groups. The level of ERα67 was significantly greater in the E group than in the other groups except at 6 months and was significantly lower in the OVX group than in the other groups except at 6 months. ERα46 was greater in the C group than in the other groups throughout the study and was always significantly lower in the OVX group than in the other groups.

Discussion

Several previous studies have shown that adult rats are a suitable animal model for studying the onset of aging (35, 36). As early as 6 months of age, rats begin to display signs of age-induced metabolic disturbances (37, 38). In addition, the prevalence of impaired glucose tolerance and type 2 diabetes mellitus has been shown to increase with age in both humans (39) and rats (37). It has been demonstrated clearly in both clinical and experimental studies that diabetes mellitus (type 1 and type 2) is an increasingly common disorder associated with several CNS complications (40, 41). Various studies have shown that estradiol administration in postmenopausal females has positive effects not only on the CNS but also on glucose homeostasis (5, 26, 28, 42–46). Taken together, these observations indicate that treatment with estradiol may be able to prevent age-related insulin resistance and neurodegenerative diseases. However, studies that have investigated the beneficial effects of low-dose 17β-estradiol on glucose homeostasis have shown that estradiol treatment does not prevent the impairments in memory and learning that are associated with the aging process (26). Despite the previous findings reported by our laboratory, we hypothesize, as do others (5, 43), that low doses of estradiol could improve some aspects of brain function that are impaired with aging.

Glucose is the primary energy source for the brain. It has been shown that Glut-3 and Glut-4 are coexpressed in the rat (47) and primate (25) brains; however, the significance of this finding is not fully understood. The first step in the present study was to determine the changes in Glut-3 and Glut-4 levels that occurred in our experimental model. Interestingly, in both brain regions that were studied, the aging process reduced the Glut-3 and Glut-4 levels from 12 months onwards, as seen in the C group (Figs. 3 and 4). This inflection point coincides with the time at which all intact animals showed irregular cycling, mainly a persistent diestrous phase. Therefore, it could be considered that this point marks the onset of a gradual decline in regular estrous cyclicity, which is associated with a gradual impairment in ovulatory function and altered patterns of steroid secretion (48–50). It is also probably associated with a gradual impairment in brain homeostasis, as shown by data that demonstrate changes in the glucose transporters.

To the best of our knowledge, this is the first paper to show the long-term effect of ovariectomy on Glut-3 and Glut-4 levels in the rat brain. Clearly, ovariectomy reduced the level of Glut-3 in both brain regions from 6 months when compared with the level in control animals (Fig. 2, A and B). Moreover, the decreased Glut-3 level was associated with the aging process, because the levels of this glucose transporter decreased significantly from 12 months in the cortex and from 6 months in the diencephalon. Glut-3 is one of the main glucose transporters and is responsible for most of the glucose uptake and utilization in the brain. Thus, early loss of ovarian function may increase the effects of aging on the brain in relation to glucose uptake and utilization. The Glut-4 levels observed in the diencephalon of the OVX group (Fig. 3B) were similar to the Glut-3 levels; therefore, if Glut-4 is an alternative glucose transporter in the brain, the combined effects of aging and early ovariectomy are similar. However, neither aging nor ovariectomy had any effect on Glut-4 levels in the cortex of OVX rats until 18 months after ovariectomy (Fig. 3A); at 12 months, the Glut-4 level in the OVX group was significantly higher than in the C group. Therefore, ovarian hormones do not appear to be involved in controlling Glut-4 levels in the cortex of middle-aged rats. The aging process, which occurs between 18 and 24 months in the rat, appears to have more important consequences on Glut-4 levels in the cortex. Currently, there is no convincing explanation for this finding.

Compared with those in the OVX group, the levels of both transporters markedly increased in the group treated with estradiol, except for the Glut-4 level in the cortex at 6 and 12 months. It is likely that the expression rate of the Glut-4 gene is down-regulated by estradiol. Progesterone may regulate the role of estradiol, based on the results seen in the C group, and the absence of both hormones implies the loss of Glut-4 gene regulation, as occurred in the OVX group. Given these results, the estradiol-induced increase in the expression of glucose transporters may improve

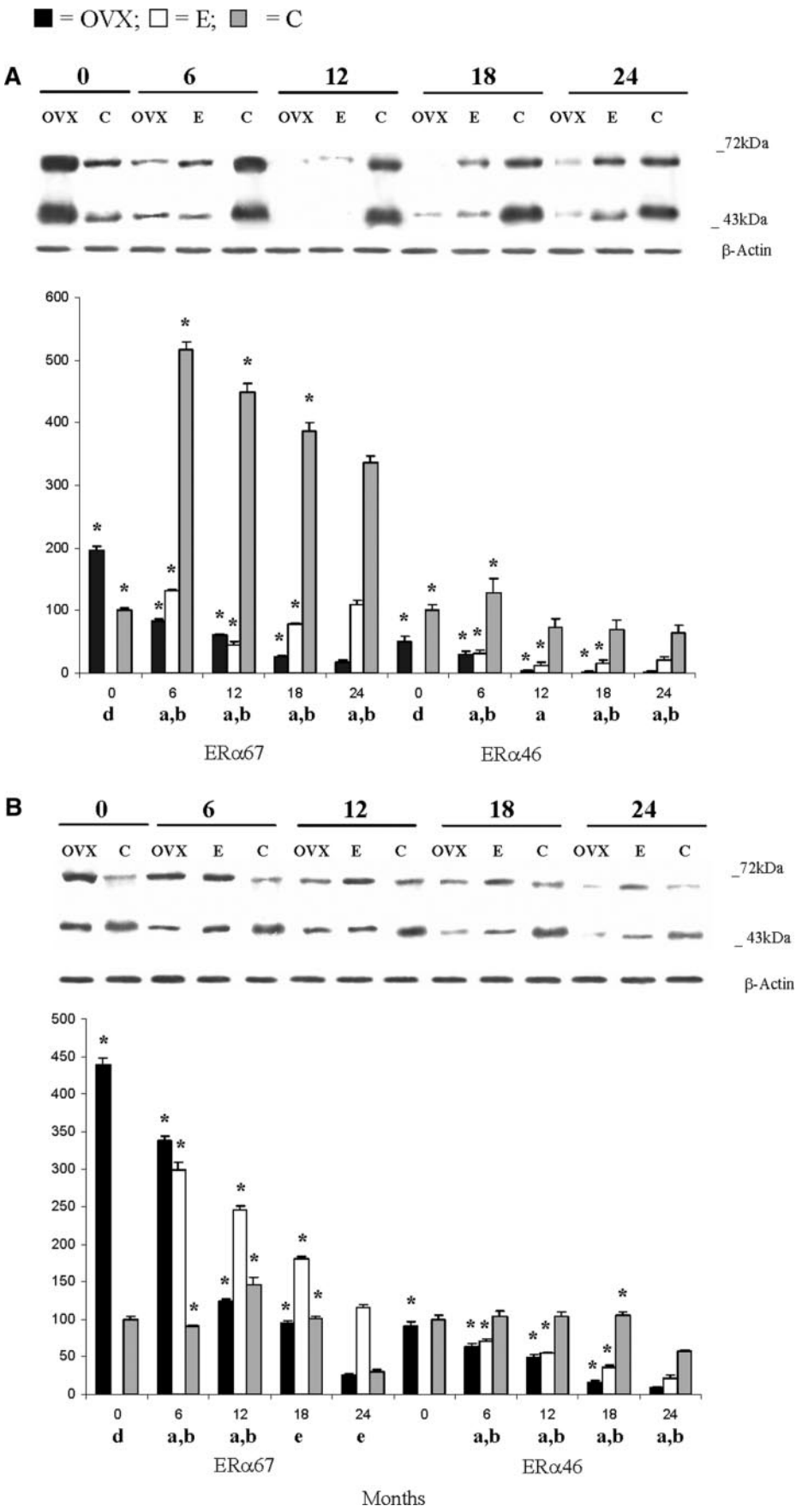


FIG. 8. ERα in cortex (panel A) from intact (C) (ERα67: $F_{(4,34)} = 141{,}550.4$; $P < 0.0001$; ERα46: $F_{(4,34)} = 38{,}307.8$; $P < 0.0001$), ovariectomized (OVX) (ERα67: $F_{(4,34)} = 720.3$; $P < 0.0001$; ERα46: $F_{(4,34)} = 2312.8$; $P < 0.0001$), and estradiol-treated (E) rats (ERα67: $F_{(4,34)} = 12{,}902.5$; $P < 0.0001$; ERα46: $F_{(4,34)} = 13{,}561.7$; $P < 0.0001$) at 0 (ERα67: $t_{(12,0.05)} = 3.56$; $P \leq 0.05$; ERα46: $t_{(12,0.05)} = 4.52$; $P \leq 0.05$), 6 (ERα67: $F_{(2,20)} = 78{,}453.8$; $P < 0.0001$; ERα46: $F_{(2,20)} = 4298.7$; $P < 0.0001$), 12 (ERα67: $F_{(2,20)} = 150{,}982.3$; $P < 0.0001$; ERα46: $F_{(2,20)} = 753{,}978.4$; $P < 0.0001$), 18 (ERα67: $F_{(2,20)} = 731{,}066.9$; $P < 0.0001$; ERα46: $F_{(2,20)} = 129{,}222.7$; $P < 0.0001$), and 24 (ERα67: $F_{(2,20)} = 67{,}316.5$; $P < 0.0001$; ERα46: $F_{(2,20)} = 45{,}450.0$; $P < 0.0001$) months of age and in diencephalon (panel B) from intact (C) (ERα67: $F_{(4,34)} = 1993.3$; $P < 0.0001$; ERα46: $F_{(4,34)} = 4552.3$; $P < 0.0001$), ovariectomized (OVX) (ERα67: $F_{(4,34)} = 6201.7$; $P < 0.0001$; ERα46: $F_{(4,34)} = 155.7$; $P < 0.0001$), and estradiol-treated (E) rats (ERα67: $F_{(4,34)} = 3918.2$; $P < 0.0001$; ERα46: $F_{(4,34)} = 8914.6$; $P < 0.0001$) at 0 (ERα67: $t_{(12,0.05)} = 51.54$; $P \leq 0.05$; ERα46: $t_{(12,0.05)} = 1.02$; P = nonsignificant), 6 (ERα67: $F_{(2,20)} = 25{,}334.1$; $P < 0.0001$; ERα46: $F_{(2,20)} = 3745.4$; $P < 0.0001$), 12 (ERα67: $F_{(2,20)} = 3470.1$; $P < 0.0001$; ERα46: $F_{(2,20)} = 8122.5$; $P < 0.0001$), 18 (ERα67: $F_{(2,20)} = 1042.4$; $P < 0.0001$; ERα46: $F_{(2,20)} = 28{,}884.6$; $P < 0.0001$), and 24 (ERα67: $F_{(2,20)} = 14{,}285.5$; $P < 0.0001$; ERα46: $F_{(2,20)} = 5332.4$; $P < 0.0001$) months of age. The histogram shows the densitometric analysis of the Western blots. Values are means ± SEM (n = 7) and represented as the percentage of control values (rats of 0 month from group C). Only significant differences are shown. Statistical significance is based on analysis of pooled raw data using the Tukey HSD. *, Month *vs.* next month; a, C *vs.* OVX, E; b, OVX *vs.* E; d, OVX *vs.* C; e, E *vs.* OVX, C.

brain glucose utilization and preserve brain function (20, 25). The results suggest that low doses of 17β-estradiol could have positive effects on rat neuronal homeostasis and could slow the progressive loss of glucose transporters that is associated with ovariectomy. It is very important to note that when the results from the E group were compared with those from the control group, the apparent amelioration of the effects of normal aging due to hormonal treatment were noticeable only in the cortex. Currently, there is no acceptable explanation for this finding, but it could have clinical relevance, because the rates of brain glucose transport and utilization are markedly reduced in elderly and diabetic patients (22). Conversely, because Glut-4 immunoreactivity has been observed in an intracellular localization, this glucose transporter could be translocated to the plasma membrane; this translocation phenomenon could be controlled by metabolic and/or hormonal parameters (for a review, see Ref. 51). Depending on the glycemia achieved during the hyperinsulinemic clamp, glucose uptake could be either increased or decreased in different brain areas (51). This could reasonably explain the different results observed for the Glut-4 levels in the cortex and the diencephalon of the various experimental groups in the current study (Fig. 3, A and B). The exact physiological role of Glut-4 in the brain is currently unknown; however, various hypotheses have been proposed; Glut-4 may provide additional glucose uptake in different brain areas or may be involved in glucose-sensing mechanisms (for a review, see Ref. 51). More investigations are needed to determine the role of estradiol in the control of brain Glut-4 levels, the physiological importance of estradiol in this role, and the possible relevance of this information to a better understanding of the effects of aging in the female brain.

Several studies have suggested that the molecular mechanism by which estradiol exerts its neuroprotective effects involves activation of the PI3-k signaling pathway (5, 9, 10, 14, 15, 52, 53). Studies have found that only high pharmacological doses of estradiol are responsible for neuroprotection (7) and activation of the PI3-k signaling pathway (9, 10). Interestingly, several studies have indicated that the improvement in insulin resistance not only improves peripheral glucose homeostasis but also improves diabetes-related CNS complications (40, 41). Our previous results have demonstrated that low-dose 17β-estradiol does not appear to ameliorate age-related effects on learning and memory (26); however, it was found that low-dose 17β-estradiol improved age-related insulin resistance in ovariectomized rats. Therefore, it is possible that our estradiol treatment model could be used to demonstrate improvements in brain homeostasis, despite our previous results on learning and memory.

ERα has been implicated in the activation of the PI3-k signaling pathway (9, 10, 14, 15), which includes key molecules of the intracellular insulin/IGF-I pathway such as IRS-1 and the p85α subunit of PI3-k. Therefore, the interactions of ERα with IRS-1 and p85α were characterized. In both regions of the brain studied here, SDS-PAGE showed two immunoreactive bands associated with IRS-1 (Fig. 4). Given their molecular masses, as indicated by SDS-PAGE, these were named ERα67 and ERα46. The existence of these two isoforms of ERα has been previously described (31, 33, 34, 41, 54, 55). Interestingly, an age-related reduction in the association between ERα67 and IRS-1 was found in both brain regions of all groups of rats in this study (Fig. 4, A and B). Therefore, 17β-estradiol treatment does not seem to prevent the age-related decrease in the ERα67-IRS-1 interaction. Compared with the C group, the ovariectomized group showed a decrease in this interaction within the cortex, and 17β-estradiol treatment did not appear to alter this finding. However, the opposite was found in the diencephalon, at least until the 12-month time point. Therefore, these results appear to support the hypothesis that progesterone has an important role in regulating the ERα67-IRS-1 interaction. More studies are required to fully investigate this issue.

In the OVX and C groups, the ERα46-IRS-1 interaction was reduced during aging in both regions of the brain that were studied, whereas estradiol treatment prevented this reduction (Fig. 4, A and B). Compared with the C group, the OVX group showed a clear reduction in this interaction. In the E group, estradiol treatment significantly increased this interaction compared with the OVX group. Therefore, these results suggest that estradiol controls the ERα46-IRS-1 interaction and appears to prevent not only the effects of ovariectomy but also the effects of the aging process.

In contrast, no interaction was detected between ERα67 and p85 in the OVX and E groups in the two regions of the brain that were studied (Fig. 6, A and B). However, this interaction was detected in the control group, which showed a significant decrease in the interaction at the end of the study (24 months) in both regions of the brain. Therefore, the ERα67-p85α interaction appears not to be exclusively controlled by estradiol; progesterone probably has an important role in the regulation of the ERα67-p85α interaction in the brain. More research is needed to prove this assumption.

Interestingly, compared with the C group, ovariectomized rats showed a reduced interaction between ERα46 and p85α (Fig. 6, A and B), and estradiol treatment only partially reversed this effect in both brain regions. Estradiol treatment appears to up-regulate this interaction during aging. The most important consequence of this effect is that it is related to an increase in the tyrosine phosphorylation rate of IRS-1, which was observed in the E group during the aging process (Fig. 5, A and B). We hypothesize that the neuroprotective properties classically ascribed to estradiol depend on the control of the ERα46-p85α interaction. ERα46α acts as an activation function-1 (AF-1) competitive inhibitor of ERα67, due, in part, to its ability to out-compete ERα67 for binding to estrogen receptors (54). It is also possible that, compared with ERα67, ERα46 has different binding affinities for coactivators or corepressors; this could also play a role in the interplay between these two isoforms and their interactions with different promoters. Moreover, estradiol was shown to down-regulate the level of ERα67 (56, 57). Therefore, it is conceivable that some actions of estradiol depend on the ERα67/ERα46 ratio through the control of ERα67 expres-

sion or stability (58). The present results suggest that the low ERα67/ERα46 ratio favors the ERα46-p85α interaction and could increase the neuroprotective effects of estradiol.

Finally, the interaction between the p85α subunit of PI3-k and IRS-1 was examined, because this interaction could play an important role in the molecular mechanism by which estradiol exerts its neuroprotective effects via activation of the IRS-1-PI3-k pathway (5, 9, 10, 14, 15). Figure 7, A and B, shows that during the normal aging process in intact rats (group C), there was a significant decrease in this interaction in both brain areas at the end of the study period (24 months; similar results were obtained in the OVX group). The early loss of ovarian function increased the effects of aging, because at 6 months in the cortex and at 12 months in the diencephalon, the interaction was significantly lower in the OVX group than in the C group. In contrast, estradiol treatment appeared to significantly increase this interaction in both brain regions from 12–24 months (Fig. 7, A and B). Estradiol treatment not only prevented the effects of ovariectomy in relation to the IRS-1-p85α interaction, but this interaction was also significantly higher in the E group than in the C group at 24 months in both regions of the brain. This appears to indicate that the increase in phosphorylation of IRS-1 observed in the E group from 6 months (Fig. 5, A and B), which was probably induced by estradiol, reflects functional activation of the IRS-1 signaling pathway. Therefore, the effect of estradiol on the interaction between p85α and IRS-1 indicates that estradiol may regulate this signaling pathway in the brain. This observation supports the theory that we have, to the best of our knowledge, proposed for the first time in this paper.

Other ligands may activate IRS-1/PI3-k pathways in the brain. In particular, IGF-I seems to be particularly important in the process of neuroprotection; it can reverse age-related effects (59) and attenuate the age-related decrease in cerebral glucose utilization (60). Moreover, gonadal hormones have been found to regulate the expression of IGF-I receptor mRNA and IGF-I-binding protein mRNA in adult female rat brains (61, 62). Several studies have shown that low doses of 17β-estradiol increase IGF-I binding in the brain by significantly increasing the numbers of IGF-I receptors (63). Therefore, it seems clear that the interaction of both systems plays a role in the prevention of neuronal age-related effects (64). The neuroprotective effect of estradiol is abolished by PI3-k inhibitors (65); this indicates that PI3-k has a role in estradiol-mediated neuroprotection and may be involved in the IGF-I receptor pathway, which is known to be activated by ERα but not by ERβ (66). After systemic administration of estradiol to adult ovariectomized rats, there is a transient increase in tyrosine phosphorylation of the IGF-I receptor in the brain, transient interaction of the IGF-I receptor with ERα but not with ERβ, and enhanced interaction of ERα with p85α (15). These findings suggest that by interacting with some components of the IGF-I signaling pathway, ERα affects the actions of IGF-I in the brain. These findings suggest future avenues of research.

However, interesting questions remain. The findings of the present study do not explain whether the increased interaction between ERα and IRS-1 in turn increases the p85α-IRS-1 interaction or whether the ERα-IRS-1 and ERα-p85α interactions are necessary to facilitate the p85α-IRS-1 interaction. It is not known whether the interactions between ERα and IRS-1 and p85α occur sequentially or whether ERα binds to a multimolecular complex of IRS-1 and PI3-k. Mendez *et al.* (15) also studied this issue. They questioned whether the ERα that interacts with this signaling pathway is localized in the cytoplasm or is associated with specific membrane domains, such as caveolae (67). In the present study, only some of the components that may be involved in the neuroprotective effect of estradiol were investigated. The effects of other important molecules, such as Akt, MAPK, and glycogen synthase kinase 3 (9, 10, 68, 69), which were not studied, should be investigated using our experimental animal model to fully understand the neuroprotective role of estradiol.

In this paper, the changes in ERα and its interactions with several proteins involved in the PI3-k pathway were studied because it has been shown, with respect to the neuroprotective effects of estradiol, that ERα, but not ERβ, plays a critical role in the treatment and prevention of neural dysfunction that is associated with normal aging or brain injury (13). Our results clearly show that normal aging (group C) was associated with a decrease in both ERα isoforms in both of the brain regions that were studied (Fig. 8, A and B) and that ovariectomy aggravates the effects of normal aging, as has already been extensively documented. However, although it was thought that estradiol treatment would reverse the effects of ovariectomy, this was observed only with respect to ERα67 in the diencephalon (Fig. 8B). Our results clearly show that 17β-estradiol treatment prevented the effects of ovariectomy on both ERα isoforms. The expectations for estradiol treatment were based on the results related to Glut-3 and Glut-4 levels in the cortex (Figs. 2A and 3A, respectively), the ERα-IRS-1 interaction (Fig. 4, A and B), and the ERα46-p85α interaction (Fig. 6, A and B); these data appear to suggest that ERα46 could be the estradiol-modulated isoform of ERα that is responsible for the mediation of the neuroprotective effects of estradiol. However, the results shown in Fig. 8, A and B, raise some doubts about the role of ERα46. It is necessary to further explore the idea that the key to the neuroprotective effects of estradiol is related to the ERα67/ERα46 ratio and that this ratio depends on the region of the brain that is studied and/or the estradiol doses used.

In summary, our findings indicate that 17β-estradiol treatment during aging in ovariectomized rats may activate the IRS-1 signaling pathway, thereby increasing the tyrosine phosphorylation of IRS-1 and the interaction between the p85α subunit of PI3-k and IRS-1. Moreover, 17β-estradiol treatment appears only to control the interactions that involve the ERα46 isoform. During aging, 17β-estradiol treatment may improve some aspects of neuronal homeostasis, because the levels of expression of the glucose transporters in both regions of the brain of estradiol-treated rats were significantly increased compared with the control group. However, more studies are required to clarify the neuroprotective role of estradiol in

relation to the other important molecules involved in the IRS-1-PI3-k signaling pathway.

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