

# Aging and substitutive hormonal therapy influence in regional and subcellular distribution of ER $\alpha$ in female rat brain

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**Abstract** Estrogens are not only critical for sexual differentiation it is well-known for the role of 17 $\beta$ -estradiol (E2) in the adult brain modulating memory, learning, mood and acts as a neuroprotector. E2 exerts its actions through two classical receptors: estrogen receptor alpha (ER $\alpha$ ) and estrogen receptor beta (ER $\beta$ ). The distribution of both receptors changes from one brain area to another, E2 being able to modulate their expression. Among the classical features of aging in humans, we find cognitive impairment, dementia, memory loss, etc. As estrogen levels change with age, especially in females, it is important

to know the effects of low E2 levels on ER $\alpha$  distribution; results from previous studies are controversial regarding this issue. In the present work, we have studied the effects of long-term E2 depletion as well as the ones of E2 treatment on ER $\alpha$  brain distribution of ovariectomized rats along aging in the diencephalon and in the telencephalon. We have found that ovariectomy causes downregulation and affects subcellular localization of ER $\alpha$  expression during aging, meanwhile prolonged estrogen treatment produces upregulation and overexpression of the receptor levels. Our results support the idea of the region-specific neuroprotection mechanisms mediated by estradiol.

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## Abbreviations

Au Cx	Auditory cortex
CA	Cornu Amonis region
CNS	Central nervous system
DAB	Diaminobenzidine
E2	17 $\beta$ -estradiol
ER $\alpha$	Estrogen receptor alpha
ER $\beta$	Estrogen receptor beta
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
M Cx	Motor cortex
MDN	Medial dorsal nucleus
M Hb	Medial habenular nucleus
MPA	Medial preoptic area

O Cx	Olfactory cortex
PBS	Phosphate-buffered saline
PVN	Paraventricular nucleus
RIA	Radioimmuno assay
S Cx	Sensorial cortex
SON	Supraoptic nucleus
V Cx	Visual cortex

## Introduction

It is known that estrogens are not only hormones critical for sexual differentiation. The primary biologically active form of estrogens, the 17 $\beta$ -estradiol (E2), plays a paramount role in the brain. During development, the brain is exposed to estradiol from different sources, and the effects of this molecule are region-dependent, promoting, or preventing apoptosis, synaptogenesis, and affecting the morphology of neurons and astrocytes (for a review see McCarthy 2008). Estrogens are known trophic factors for neurons. Synapses remodeling is a phenomenon that fluctuates according to hormone levels during the estrous cycle (Olmos et al. 1987). Other areas where growth-promoting effects of the estrogens have been proven are the midbrain (Reisert et al. 1987), cortex (Garcia-Segura et al. 1989), pituitary (Chun et al. 1998), and spinal cord (VanderHorst and Holstege 1997).

In the adult brain, E2 modulates learning, memory, mood (McEwen 2002), and even auditory physiology (Tremere et al. 2009). Estrogens are also able to prevent neuronal death in several models of brain injury (Liu et al. 2010), to ameliorate brain inflammation (Pozzi et al. 2006), and to be implicated in many neurological disorders as Parkinson's disease (Baraka et al. 2011) and Alzheimer's disease promoting the nonamyloidogenic metabolism of the amyloid precursor protein (Jaffe et al. 1994).

Estrogens exert their actions mainly through two different receptors. Estrogen receptor alpha (ER $\alpha$ ), the first to be discovered and cloned (Jensen 1962; Walter et al. 1985), is a 66-Kda protein in its most common form; estrogen receptor beta (ER $\beta$ ) was discovered a decade later (Mosselman et al. 1996) but both of them present different splicing variants as well as natural occurring mutants.

In the rat brain, ER $\alpha$  is detected at high levels in areas related with sexual behavior as hypothalamic,

preoptic, and limbic structures, but it is also found in olfactory regions, cerebellum, area postrema, and substantia gelatinosa of the spinal cord (Simerly et al. 1990; Laflamme et al. 1998; Belcher 1999). In spite of both classical receptors being expressed in the brain, ER $\beta$  seems to be distributed in a wider pattern (Belcher 1999; Laflamme et al. 1998; Osterlund et al. 1998, 2000). The general distribution of estrogen receptor (ER) mRNA throughout the brain is quite similar in different species, but the differences in the expression pattern are important enough for making data extrapolation inadequate. For example, human paraventricular nucleus expresses both classical receptors, with ER $\alpha$  being predominant, while only ER $\beta$  has been found in this area in the rat brain (Shughrue et al. 1997; Küppers and Beyer 1999; Gundlach et al. 2000; Osterlund et al. 2000).

Estrogen-mediated neuroprotection can take place through two different pathways. One way involves canonical ER activation, which is constitutively expressed in many brain regions and is able to initiate gene transcription after specifically binding to estradiol (Green and Simpkins 2000; Marin et al. 2005). The second way that has been growing interest and refers to the rapid non-genomic (or alternative) signaling pathways, involves extranuclear ER in response to physiological concentration of estrogens to elicit neuroprotection (Singer et al. 1999; Adams et al. 2002; Mendez et al. 2003; Simoncini et al. 2003). Recent studies point to a coupling between both mechanisms (for review see Vasudevan and Pfaff 2008).

Due to their neuroprotective effects, the use of estrogens has been proposed for the treatment of different neuropathologies as Alzheimer's disease (Amtul et al. 2010), Parkinson's disease (Baraka et al. 2011), ischemic stroke (Dang et al. 2011), schizophrenia (Kulkarni et al. 2010), and Huntington's disease (Túnez et al. 2006). However, some epidemiological studies show no benefits on cognitive performance after hormone therapy (Coker et al. 2010; Mulnard et al. 2000; Shumaker et al. 2004), so the subject is very controversial.

It is well established that with increasing age, several aspects of brain function are impaired in rodents, non-human primates, and humans, including deficits in learning and memory, neurogenesis, neuronal, and synaptic density and alterations in dendritic architecture (Sherwin 1994, 1997, 2003). As we discussed above, ER $\alpha$  has a distribution that varies among the different cerebral areas studied, as well as with different situations. Because of the potential importance of

this receptor in the mediating effects of changing estradiol levels with aging, many studies achieved the amount of global immunostaining and distribution of ER $\alpha$  in several brain areas, such as the hippocampus (Adams et al. 2002; Mehra et al. 2005) or hypothalamus (Funabashi et al. 2000). Furthermore, it was reported that not only is there an age regulation of the intensity of immunostaining, but also an evident influence of aging in the subcellular distribution of this receptor (Ishunina et al. 2000; Milner et al. 2001; Kalesnykas et al. 2005). In ovariectomized animals, the effect of the sexual hormones in the ER $\alpha$  level and its localization within the cell, as is described by many researchers has also been widely demonstrated (Adams et al. 2002; Wilson et al. 2002; Stirone et al. 2003).

Previous studies have produced conflicting results concerning the ability of estrogen to regulate ER protein expression in the brain. To elucidate the long-term effect of ovariectomy and 17 $\beta$ -estradiol treatment on the profile of ER $\alpha$  in the brain, we performed a study of the ER $\alpha$  distribution and quantified the protein signal in some selected brain areas using immunohistochemistry for ER $\alpha$ . These areas, cerebral cortex and diencephalon, are recognized targets of ovarian hormone stimulation. We have shown that ovariectomy causes downregulation and different subcellular localization of ER $\alpha$  expression during aging, meanwhile prolonged estrogen treatment produces upregulation and overexpression of the receptor levels.

## Materials and methods

### Animals

Virgin female Wistar rats (from the Biotery of the Faculty of Medicine, University of Oviedo), weighting 250–280 (age, 8–10 weeks), kept under standard conditions of temperature (23 $\pm$ 3 $^{\circ}$ C) and humidity (65 $\pm$ 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 which were carried out with the 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

The animals 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 17 $\beta$ -estradiol (E) and sham surgery animals (intact) (C), and housed individually throughout the experiment.

After surgery, all the rats began the experimental treatment exactly 1 week after ovariectomy to ensure a uniform time of estrogen depletion before replacement and to recover from surgery stress. After this, the rats were implanted subcutaneously in the posterior neck with 90-day-release 17 $\beta$ -estradiol pellets (25  $\mu$ g/pellet; Innovative Research of America, Sarasota, FL) or placebos containing no estradiol. Every 90 days, the pellets were replaced. This dosing regimen has resulted in physiological levels of plasma estradiol and has been shown to be neuroprotective in rats (Gonzalez et al. 2000).

Groups (OVX, E, and C) were divided randomly into four subgroups (ten 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 approximately 8, 14, 20, and 26 months old. Moreover, 14 animals (seven OVX and seven C) were killed 1 week after ovariectomy (age, 9–11 weeks). Therefore, the animals included in this group did not receive any treatment and were considered as OVX-0 months and C-0 months 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. After month 12 of the experiment, none of the intact rats showed repetitive estrous cycles, instead, 87.26 % of animals showed persistent diestrous phase.

Blood samples for the determination of 17 $\beta$ -estradiol plasma concentrations were collected from the jugular vein into heparinized tubes, centrifuged at 3,000 rpm for 20 min at 4 $^{\circ}$ C, and plasma was immediately drawn off and stored frozen at –20 $^{\circ}$ C until assayed. Plasma 17 $\beta$ -estradiol was measured by RIA using Immunoche kits of cover tubes (ICN Biomedicals, Inc.). The assay sensitivity was 10 pg/ml, and the intra-assay coefficient of variation was 9.45 %. All samples were measured on the same day. The sample was assayed in triplicate.

## Histology, immunohistochemistry, and morphometric analysis

The rats of each subgroup were perfused, under halothane inhalation anesthesia, through the left ventricle, first with 100 ml of 0.9 % NaCl and then with 300 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and post-fixed by immersion in the same fixative overnight and then rinsed in PBS.

After fixation, the brains were washed in distilled water, dehydrated through successive alcohols, cleared in two baths of butyl acetate, embedded in paraffin, and blocked out in a suitable mold. Transverse sections about 10- $\mu$ m thick were obtained and attached to gelatin-covered slides. Sections were deparaffined in xylene and rehydrated, and an immunocytochemical method was carried out for detection of ER $\alpha$ . The sections were treated sequentially with Triton X (0.1 %, 5 min) at room temperature (RT), washed in distilled water, treated with H<sub>2</sub>O<sub>2</sub> (3 %, 5 min) in a wet chamber at RT, washed in distilled water and treated with phosphate-buffered saline (PBS) (2 min). Slides were placed in a plastic Coplin jar filled with 0.01 M sodium citrate buffer (pH 6), and incubated in a household microwave oven four times for 2 min each operating with the highest power setting (800 W). Non-specific binding was blocked by incubation with bovine serum. Incubation with a specific antibody against ER $\alpha$  (sc-542, Santa Cruz Biotechnology, Inc., diluted 1:1,000) was performed overnight at 4°C. After several washes in PBS, the sections were incubated for 30 min at RT using a biotinylated horse universal antibody (Vector, PK-8800) diluted 1:50. Afterwards, the sections were incubated with Extravidin (Sigma Extra-3) labeled with HRP. The peroxidase activity was visualized by incubation with 0.05 % DAB (Sigma, D5637-25 G) in 50 mM Tris buffer pH 7.6, containing 0.04 % H<sub>2</sub>O<sub>2</sub> (33 %). The usual specificity control tests were carried out.

After the immunohistochemistry, sections were counterstained with a modification of formaldehyde thionin method (Tolivia et al. 1994), dehydrated, cleared in eucalyptol, and mounted with Eukitt (O Kindler GmbH & Co, Freiburg, Germany).

For the identification of neuronal nuclei, we followed the nomenclature described in: Atlas of The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 1997). After acquisition with a digital camera (Nikon

DN100) adapted on a Nikon Eclipse E400 microscope, images were processed according to the method described by Tolivia et al. (2006) to quantify the signal of the ER $\alpha$  immunostaining. After acquisition with a digital camera, images are opened in Adobe Photoshop. For subsequent comparison of the amount present in different sections, the magnification of images must be equal. To select the specific signaling, choose “Color range,” in the “Select menu” of Photoshop, and with the “Eyedropper” tool click on any object on the image displaying the desired color/chromogen and all areas with the selected color range will be highlighted in an automatically generated clone image; thus the selection process can be controlled at every step. The tolerance bar of the color range tool makes the direct visualization of the selection on the image possible, and minimal variations in the chromogen selection are clearly observed. Once chromogen selection is finished, the profile used can be archived and used in the future when similar marked sections are studied. Close the “Color range” panel, and all immunopositive objects in the original image appear highlighted. Open the “Edit” menu of Photoshop, select “Copy,” open a “New file” and “Paste” the selected image. The new image shows only the positive selected areas on a white background. Convert the image to gray; the new picture shows all selected marked areas in various gray tones, which reflects both the positive area of the signal and the intensity of it (i.e., the amount of the chromogen present in each region). This gray picture must be saved in TIF format, which can be opened without problem by the Scion Image program (other free image analysis programs, as Image J, can be used). Open the picture in this program and calculate the “Mean density” under “Uncalibrated” conditions. The measurements obtained show the strength signal of the image and can be copied and exported to a spreadsheet (i.e., Excel). The strength signal obtained varies between 0 and 255 (value 0 corresponds to a nonstained section [white], and value 255 corresponds to a fully stained section [black]).

## Statistical analysis

Data are expressed as mean $\pm$ standard error of mean. First, we evaluated the Gaussian distribution of each variable by a Kolmogorov–Smirnov test and the aleatoricity of the samples by a Rachas test. Thereafter, data were statistically analyzed using an analysis of

variance design followed by between-group comparison using the Tuckey honestly significant difference test. Values of  $P \leq 0.05$  were considered significant.

## Results

### General characteristics of experimental animals

Plasma  $17\beta$ -estradiol values obtained during the study were previously published in recent papers of our group (Moreno et al. 2010; Pérez et al. 2011). The plasma level of  $17\beta$ -estradiol was significantly higher in groups E and C than in group O at all time points. In addition, plasma levels of  $17\beta$ -estradiol were significantly higher in group E than in group C at 18 and 24 months. In the C group animals, plasma levels of  $17\beta$ -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 group O during the first 6 months (Fig. 1).

### ER $\alpha$ presence in the telencephalon

The densitometric study of the whole telencephalon shows differences in the ER $\alpha$  distribution pattern that can be observed among the three experimental groups. While in the younger animals of the C group ER $\alpha$  is mainly localized in the neuronal nucleus, perinuclear immunostaining is observed in the OVX and E groups. The C group shows a clear modification in the distribution of ER $\alpha$  during aging; the immunostaining appears also in the cytoplasm as well as in the nucleus. The quantification of the immunostaining noted an

increase of ER $\alpha$  presence with aging in the C and E groups while in the OVX group a decrease in the expression of ER $\alpha$  was observed (Fig. 2).

Due to the heterogeneity and complexity of the telencephalon and for better analysis purposes, we divide it in the following areas of study: hippocampus, olfactory cortex and motor, sensorial, visual, and auditory cortices.

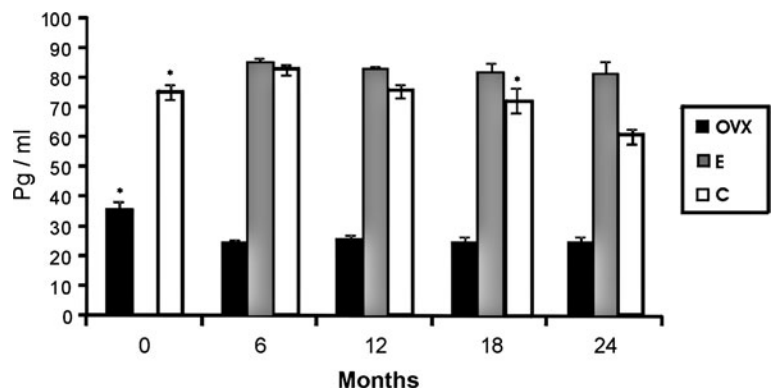
### ER $\alpha$ in the hippocampus (Hp)

The immunopositivity for ER $\alpha$  was located mainly in the nucleus of pyramidal neurons of the CA1, CA2, CA3, and in the granular neurons of the dentate gyrus. Some neurons of these areas also show a slight extranuclear staining (Fig. 3a–f). During aging, the extranuclear staining for ER $\alpha$  mainly appears in the pyramidal neurons of the CAs of OVX and C groups (Fig. 3e). In the E group, the immunoreactivity along aging is principally located at nuclear level (Fig. 3f). The quantification of the immunostaining noted an increase of ER $\alpha$  presence with aging in the E group while in the OVX group a deep decrease in the expression is observed. In the C group, there is no change in receptor levels of ER $\alpha$  (Fig. 4a).

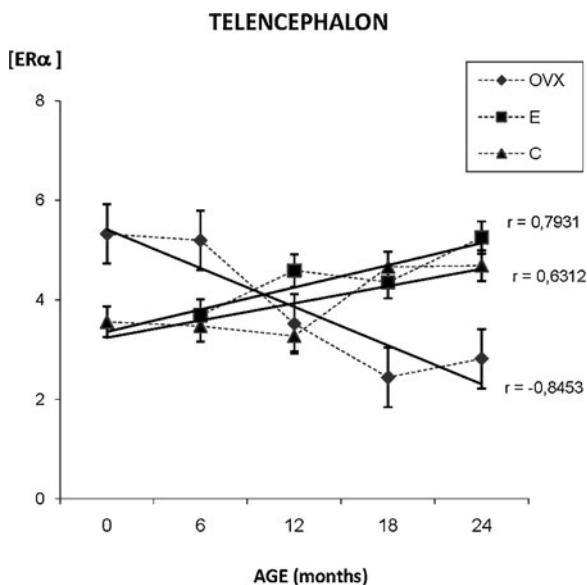
### ER $\alpha$ in the frontal motor cortex

The neurons of the motor cortex show an ER $\alpha$  nuclear positive staining, mainly in the external pyramidal layer of all groups (Fig. 3h, l). Related with aging, the external pyramidal and granular layer neurons show a cytoplasmic staining in the youngest animals of all experimental groups. This homogeneity in ER $\alpha$  distribution changes with aging when the signal in the granular layer in the E and C groups virtually disappears. Group E is

**Fig. 1** Levels of  $17\beta$ -estradiol of ovariectomized animals (O; black bars), sham surgery animals (intact) (C; white bars) and ovariectomized animals treated with  $17\beta$ -estradiol (E; gray bars). Mean  $\pm$  standard error of the mean for seven animals. Significant differences are shown. \* $p \leq 0.05$  (month vs. next month)







**Fig. 2** Densitometric quantification of ER $\alpha$  immunosignal in the telencephalon along aging in the three experimental groups: control (C), ovariectomized (OVX), and estradiol treated (E) animals, in different age groups (months). Each point in the graph represents mean density in a  $\times 20$  field  $\pm$  standard error of the mean. Regression lines and Pearson's correlation coefficient ( $r$ ) are also shown

the only group, in which the ER $\alpha$  increases with age, presenting the lowest amount of receptor in the youngest animals and the highest in the oldest. The quantification of the immunostaining noted a decrease in the expression of ER $\alpha$  with aging in the C and OVX groups (Fig. 4b).

#### ER $\alpha$ in the frontal sensorial cortex (S Cx)

The ER $\alpha$  distribution in this area is quite similar to the one in the frontal motor cortex with the receptor located mainly in the nucleus of the pyramidal neurons in the three experimental groups. The main difference we found is that during aging, in the OVX group, the nuclear ER $\alpha$  staining adopts a granular appearance instead of the homogeneous aspect showed in the young animals. On the contrary, groups E and C do not show changes regarding the deposit shape, keeping an even look all over the experiment. In the S Cx, group C shows the highest ER $\alpha$  levels in all range of ages. The quantification of the immunostaining noted a decrease in the expression of ER $\alpha$  with aging in the C and OVX groups, while in the E group, an increase of ER $\alpha$  presence was observed (Fig. 4c).

#### ER $\alpha$ in the olfactory cortex

In the olfactory cortex, the ER $\alpha$  is mainly located in the nucleus of the neurons. During aging the staining pattern is quite homogenous in both, C and E groups, and remain at nuclear level, but in the OVX, the location of the ER $\alpha$  changes with age (from nuclear to cytoplasmic) and turns more heterogeneous (Fig. 3j). This experimental group shows the lowest ER $\alpha$  levels throughout the experiment. In both groups, OVX and C, a decrease of ER $\alpha$  staining with age is observed, while there is no change in receptor levels in the E group (Fig. 5a).

#### ER $\alpha$ in the visual cortex

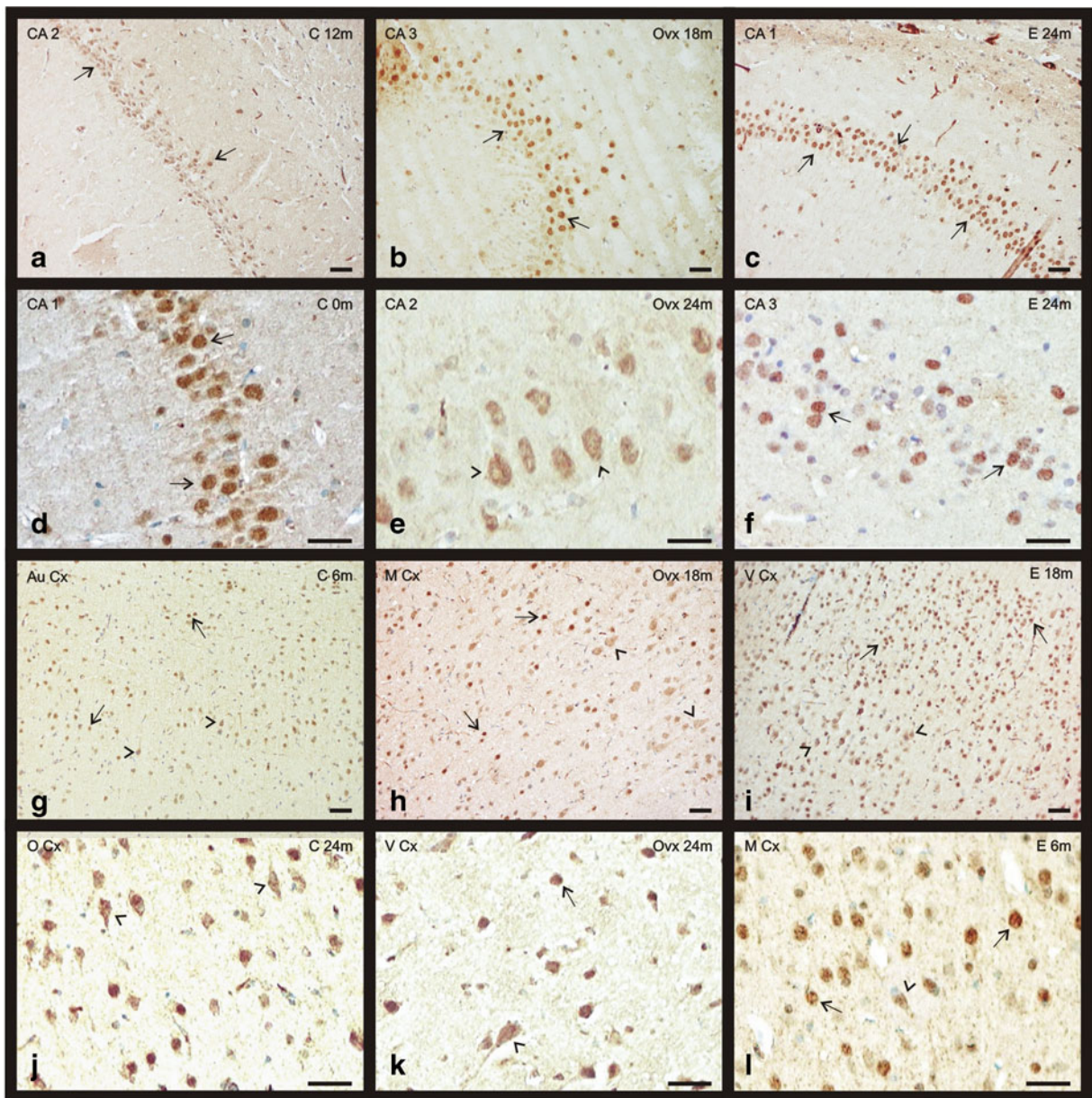
The most reactive part of this cortex is the deepest half characterized by the presence of medium-big size granular and pyramidal neurons. The granular neurons show only nuclear staining for ER $\alpha$  independently of the experimental group while the big pyramidal neurons present nuclear and cytoplasmic staining for the receptor. During aging, most of ER $\alpha$  of the pyramidal neurons is located in the cytoplasm; especially in the E group (Fig. 3i). The OVX aging animals showed few ER $\alpha$  positive neurons, a common feature of different cortical areas for 24 months animals of this experimental group (Fig. 3k). The quantification of the immunostaining noted a decrease in the expression of ER $\alpha$  related with aging in the three experimental groups: C, OVX, and E (Fig. 5b).

#### ER $\alpha$ in the auditory cortex

The ER $\alpha$  staining was located again mainly in the nucleus of pyramidal neurons of the deepest layers of the cortex. During aging, and in all the experimental groups, the immunosignal for ER $\alpha$  extends to the cytoplasm of the neurons (Fig. 3g). An increment of the receptor, related with age, was observed in the groups C and E while the OVX ones showed a decrease in the levels of ER $\alpha$ . The group E shows higher ER $\alpha$  levels at all points of age (Fig. 5c).

#### ER $\alpha$ presence in the diencephalon

The densitometric study of the whole diencephalon shows the next results. In the OVX group, we have found a global decrease on ER $\alpha$  immunostaining

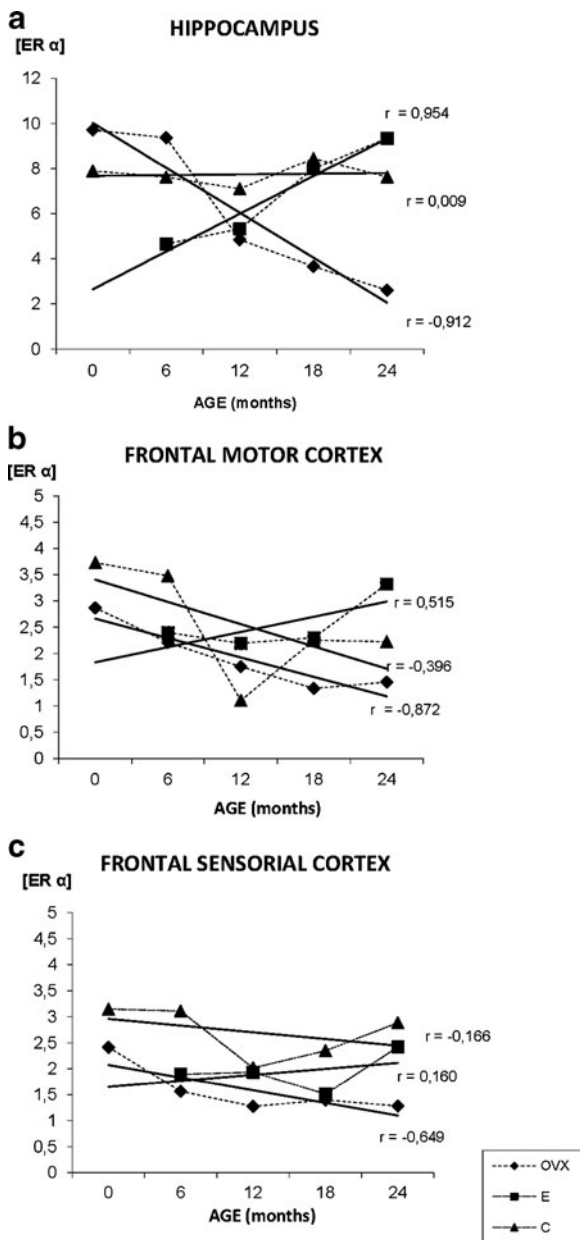


**Fig. 3** Immunohistochemical staining for ER $\alpha$  in the telencephalon of the three experimental groups: control (C), ovariectomized (OVX), and estradiol treated (E) animals during aging (0–24 months). **a–f** ER $\alpha$  presence in CAs of the hippocampus.

**g–l** ER $\alpha$  presence in some cortical regions: the auditory cortex, motor cortex, visual cortex, and olfactory cortex. Nuclear location of ER $\alpha$  (arrows). Cytoplasmic location of ER $\alpha$  (arrowheads). (bar=200  $\mu$ m) (mmonths)

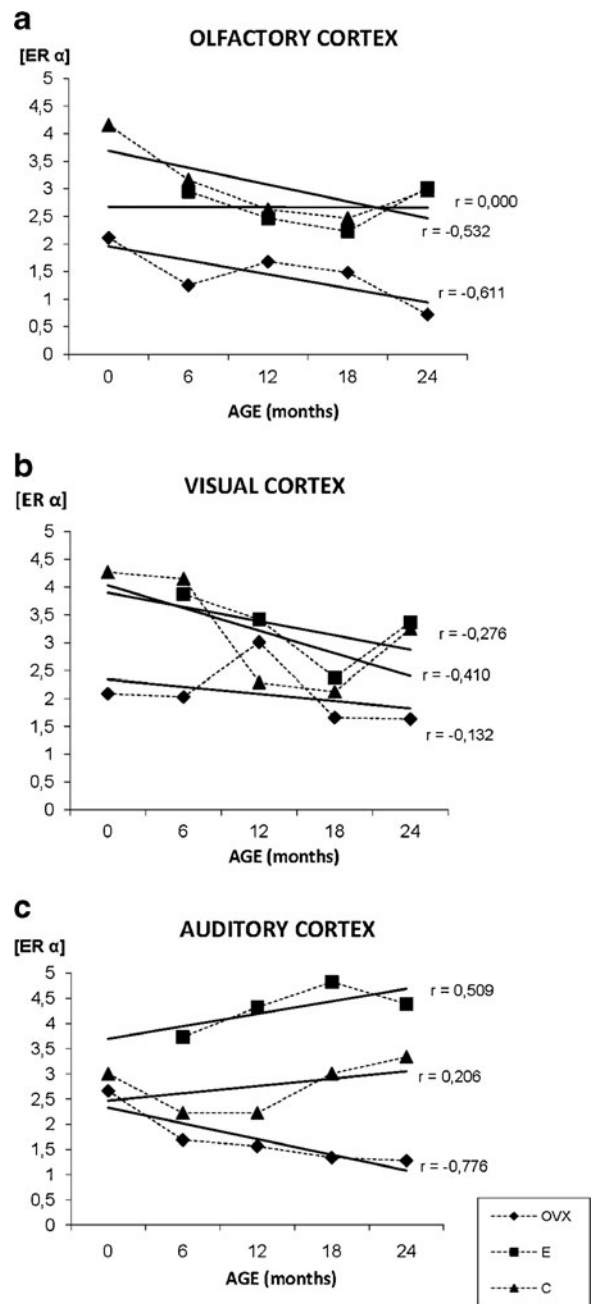
during aging process. In the E group, our results showed that estradiol treatment determines an increase on ER $\alpha$  in diencephalon while in C group, although estrogen receptor immunostaining seems to increase during aging, the lineal correlation does not confirm dependence between age and labeling intensity (Fig. 6).

In contrast to telencephalon, ER $\alpha$  immunopositivity was mainly nuclear in most of the studied areas, with the exceptions that will be pointed below. As was previously achieved in the telencephalon, for better analysis purposes the diencephalon was divided in the following representative areas for the immunohistochemistry study. The medial preoptic area (MPA),



**Fig. 4** Densitometric quantification of ER $\alpha$  immunosignal in the hippocampus and frontal cortex along aging in the three experimental groups: control (C), ovariectomized (OVX), and estradiol treated (E) animals, in different age groups (months). Each point in the graph represents mean density in a  $\times 20$  field  $\pm$  standard error of the mean. Regression lines and Pearson's correlation coefficient ( $r$ ) are also shown

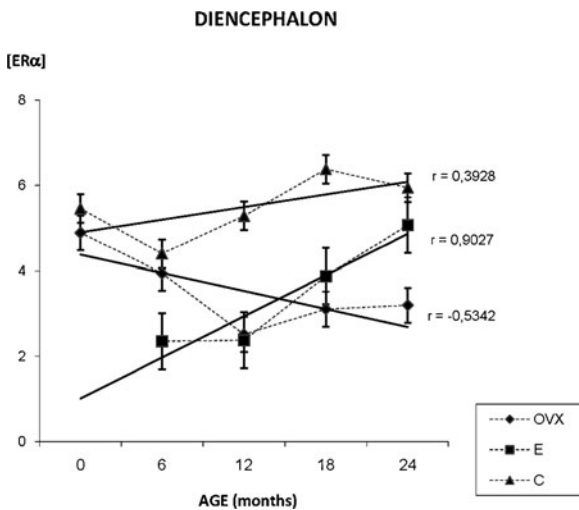
the supraoptic nucleus (SON), and the paraventricular nucleus (PVN) at hypothalamic level; the medial dorsal nucleus (MDN) of the thalamus and the medial habenular nucleus (M Hb) of the epithalamus. The observation of a different immunostaining pattern in



**Fig. 5** Densitometric quantification of ER $\alpha$  immunosignal in the olfactory, visual and auditory cortex along aging in the three experimental groups: control (C), ovariectomized (OVX), and estradiol treated (E) animals, in different age groups (months). Each point in the graph represents mean density in a  $\times 20$  field  $\pm$  standard error of the mean. Regression lines and Pearson's correlation coefficient ( $r$ ) are also shown

these areas raises the idea of a region-specific expression of ER $\alpha$ .





**Fig. 6** Densitometric quantification of ER $\alpha$  immunosignal in the diencephalon along aging in the three experimental groups: control (C), ovariectomized (OVX), and estradiol-treated (E) animals in different age groups. Each point in the graph represents mean density in a  $\times 20$  field  $\pm$  standard error of the mean. Regression lines and Pearson's correlation coefficient ( $r$ ) are also shown

#### ER $\alpha$ in the hypothalamus

Nuclear staining for ER $\alpha$  is only observed in the MPA of young animals, but from 18 months to the end of the study at 24 months, the receptor is also located in the cytoplasm, especially in the group C (Fig. 7a–c). The quantification of the immunostaining noted an increase of ER $\alpha$  presence with aging in the three experimental groups, but this increase was not statistically significant in the OVX group that always shows the lowest levels of the receptor (Fig. 8a). The group E shows the highest ER $\alpha$  levels throughout the study, but in the young animals, all values are quite similar (Fig. 8a).

The immunosignaling for ER $\alpha$ , in the PVN, is located exclusively at nuclear level of the neurons, and this location does not change with aging (Fig. 7d–f). The OVX group shows few positive neurons with a weak signal, in contrast, the E group shows the highest number of immunostained cells (Fig. 7f). During aging, the quantification of the immunostaining shows an increase of ER $\alpha$  in the C group, while a decrease in the OVX animals is noted. The values observed in group E show a slight increase with aging (Fig. 8b).

The distribution of the immunosignaling for ER $\alpha$ , in the SON, is similar in all to that described for PVN, and the location of the immunostaining does not change with aging. During aging, the quantification of the

immunostaining shows an increase of ER $\alpha$  in the C and E groups, while an absence of changes in the OVX animals was noted (Fig. 8c).

#### ER $\alpha$ in the thalamus

The number of immunopositive neurons for ER $\alpha$  in the MDN is lower when compared with other diencephalic studied areas (Fig. 7g–i). In the young animals, the receptor is located in the nucleus, but in the oldest, the immunoreactivity appears located in both, neuronal nuclei and cytoplasm, especially in the OVX group (Fig. 7h). The quantification of the immunostaining shows that there is no correlation between age and ER $\alpha$  in both, the C and E groups and only a slight decrease of the receptor with age in the OVX animals (Fig. 9a).

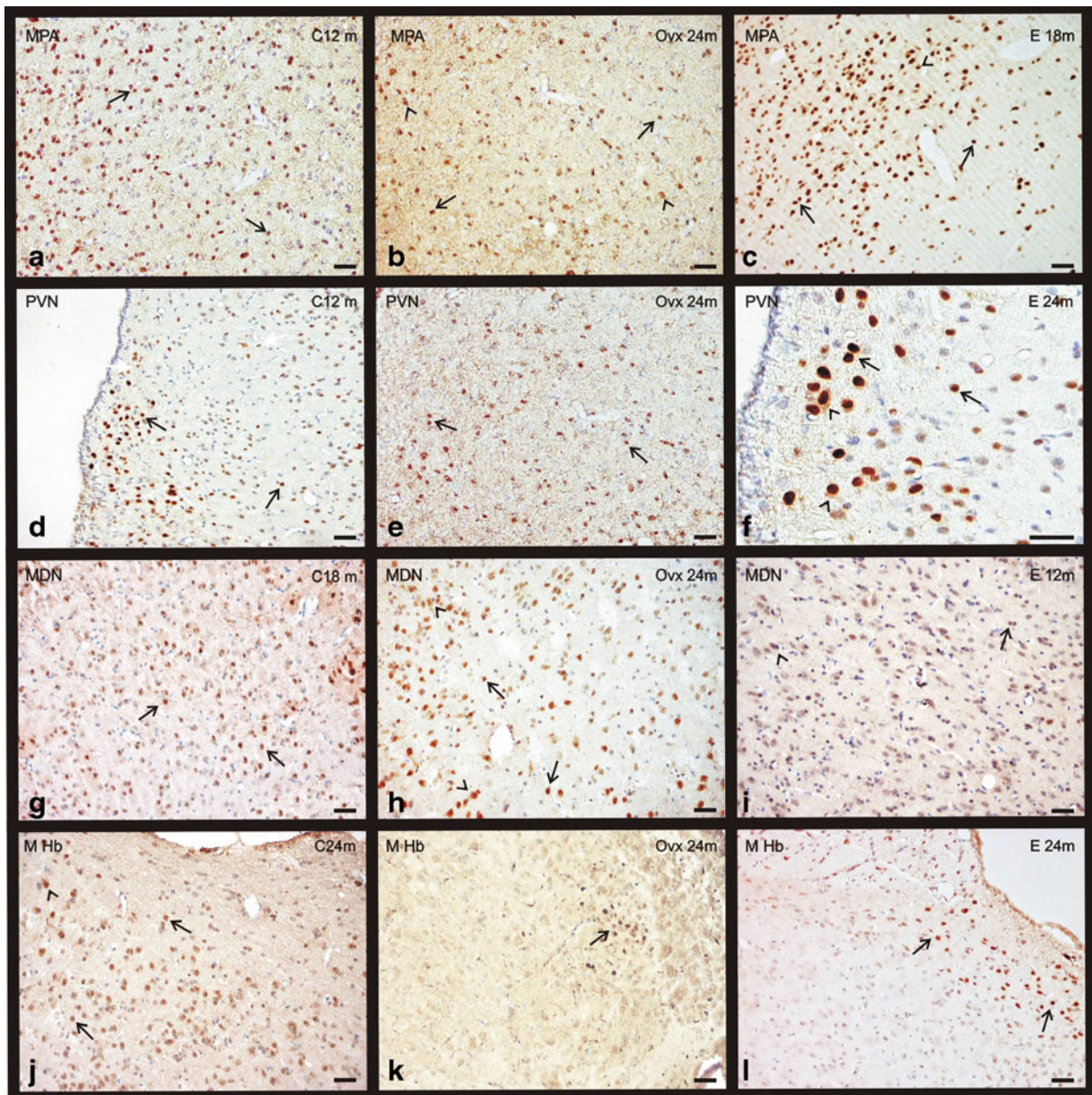
#### ER $\alpha$ in the epithalamus

The immunostaining for ER $\alpha$ , in the MHb, is mainly nuclear in all experimental groups. This nuclear location does not change with aging (Fig. 7j–k). The quantification of the immunostaining shows that there is no correlation between age and ER $\alpha$  in any of the groups, but at 12 months, the C and E groups show an important decrease in ER $\alpha$  levels (Fig. 9b). The OVX group shows the lowest values for the receptor.

## Discussion

Our results show that estrogen and aging have an effect in the distribution of ER $\alpha$  within the cell. In general, the subcellular distribution of ER was different in the three groups studied. Thus, whereas in young animals of C group most of ER immunoreactivity was concentrated into neuronal nucleus, in OVX and E groups the immunostaining not was only nuclear but also cytoplasmic. On the other hand, aging increased the cytoplasmic presence in all groups studied. As we discuss here, the subcellular distribution of ER $\alpha$  could be related with the different neuroprotection mechanisms mediated by estrogens.

In this work, we have studied ER $\alpha$  expression in two selected encephalic areas, telencephalon, and diencephalon, since this type of receptor plays a pivotal role in the maintenance of neuronal integrity in normal aging as well as in neurodegenerative diseases and injuries (Wilson et al. 2002). This function is directly related to estradiol mediated nongenomic mechanisms of action with a direct



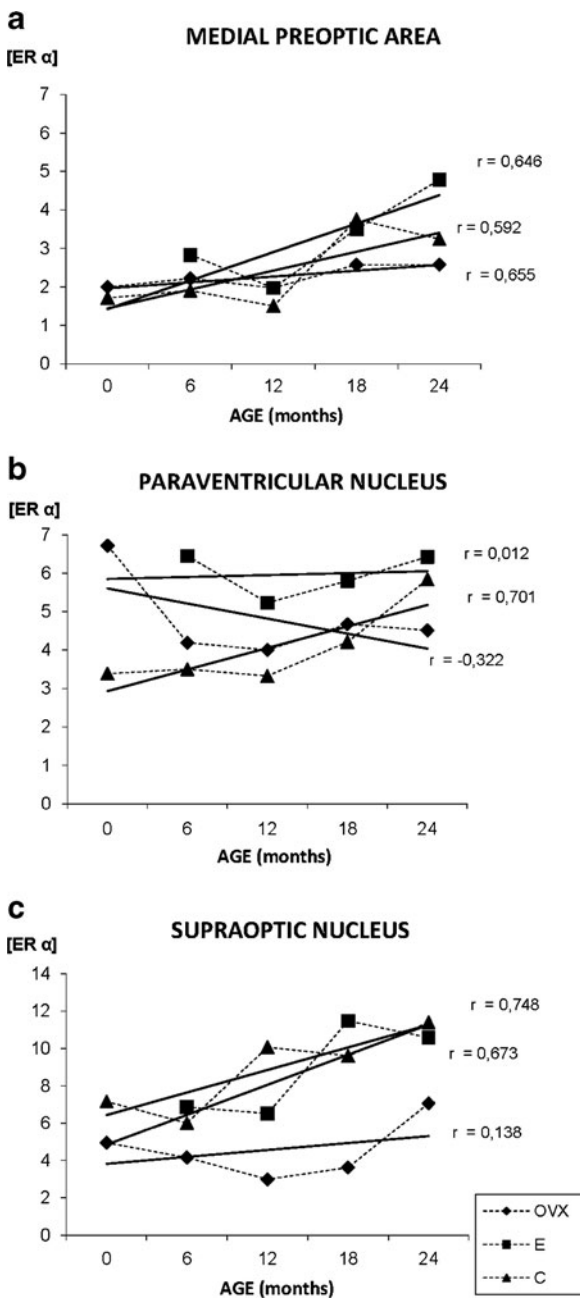
**Fig. 7** Immunohistochemical staining for ER $\alpha$  in the diencephalon of the three experimental groups: control (C), ovariectomized (OVX), and estradiol-treated (E) animals in different age groups (0–24 months). **a–c** ER $\alpha$  presence in the medial preoptic area (MPA). **d–f** ER $\alpha$  presence in the paraventricular nucleus

(PVN). **g–i** ER $\alpha$  presence in the medial dorsal nucleus (MDN). **j–l** ER $\alpha$  presence in the medial habenular nucleus (M-Hb). Nuclear location of ER $\alpha$  (arrows). Cytoplasmic location of ER $\alpha$  (arrowheads). (bar=200  $\mu$ m) (m months)

association between ER $\alpha$  and G proteins (Pedram et al. 2006). Confocal microscopy and Western blot studies have revealed the presence of many ER $\alpha$  isoforms in cerebral blood vessels (Stirone et al. 2003); among which there is an 82-KDa band corresponding to those of 80 KDa. Unlike our working group, the antibodies

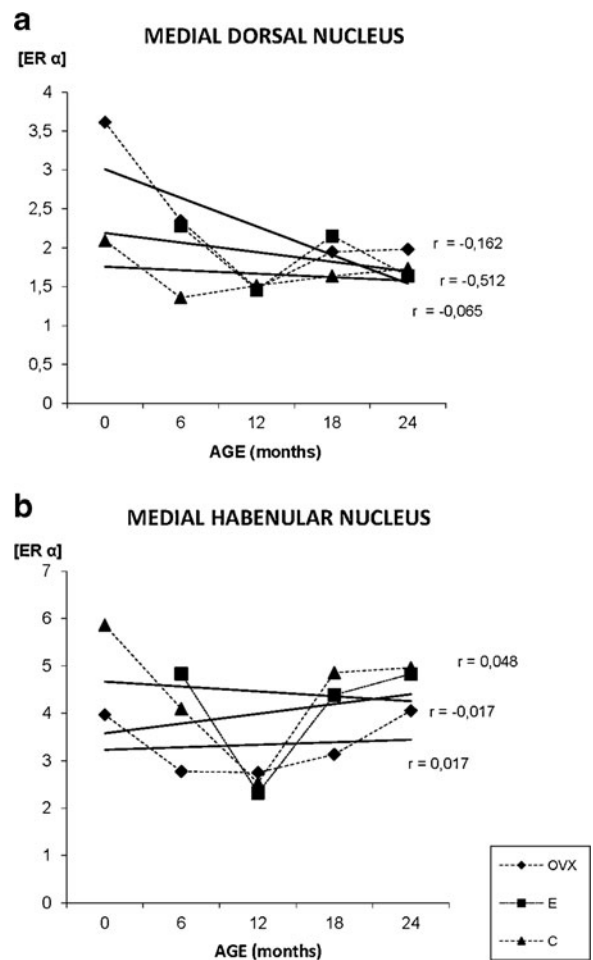
employed by these authors are generated against high variability regions of ER $\alpha$ , the H-20, which recognizes the C-terminal region, and the H-184, which binds to N-terminal region, so we are talking about monoclonal antibodies, which recognize less specific regions, which leads to different band patterns, sometimes unspecific.





**Fig. 8** Densitometric quantification of ER $\alpha$  immunosignal in the medial preoptic area, paraventricular, and supraoptic nuclei along aging in the three experimental groups: control (C), ovariectomized (OVX), and estradiol treated (E) animals in different age groups (months). Each point in the graph represents mean density in a  $\times 20$  field  $\pm$  standard error of the mean. Regression lines and Pearson's correlation coefficient ( $r$ ) are also shown

According to our previous results (Alonso et al. 2008), there is an age-dependent decrease in the 67 and 47 kDa isoforms of ER during aging process in



**Fig. 9** Densitometric quantification of ER $\alpha$  immunosignal in the medial dorsal nucleus of the thalamus and habenular nucleus of the epithalamus along aging in the three experimental groups: control (C), ovariectomized (OVX), and estradiol treated (E) animals, in different age groups (months). Each point in the graph represents mean density in a  $\times 20$  field  $\pm$  standard error of the mean. Regression lines and Pearson's correlation coefficient ( $r$ ) are also shown

telencephalon as well as in diencephalon. Ovariectomy, with the consequent loss of ovarian estrogens, aggravates the effect of normal aging in cognitive decline and compared to hormone treated animals (Cheng et al. 2001; Oge et al. 2003; Stirone et al. 2003), raising the idea that these sexual hormones exert neuroprotective effects even once menopause has occurred (Iossa et al. 1999). Thus, we have found a different pattern of ER $\alpha$  localization in telencephalon and diencephalon in the three experimental treatments. In telencephalon, in young animals from the three experimental situations, the immunostaining is mainly nuclear and aging involves a change in ER $\alpha$

localization within the cell from the nucleus to extranuclear sites. In cortex, most of the immunoreactivity for ER $\alpha$  was concentrated in small and medium-sized granular and pyramidal neurons in outer layers at nuclear and even sometimes extranuclear level; in the oldest animals, the extranuclear staining was more frequent and mainly present in pyramidal neurons, in agreement with Shughrue and Merchenthaler's results (Shughrue and Merchenthaler 2000a) and Kritzer's studies (Kritzer 2002). A similar feature occurs in the hippocampus. During the first half of the life, ER $\alpha$  tends to concentrate exclusively in nuclei of CA1, CA2, and CA3 pyramidal neurons and in some granular cells from the dentate gyrus, as it has been described by some authors (Shughrue and Merchenthaler 2000a, b; Mehra et al. 2005). From 12 months of experimentation, concomitant with the estropause, the extranuclear isoform begins to be more abundant (Pappas et al. 1995; Milner et al. 2001). Ovariectomy not only involves a decrease in immunohistochemical signal, but also leads to a noticeable extranuclear immunolabeling even more intense and earlier than in the E group. In this sense, *in vitro* studies have demonstrated that in cells showing artificial senescence, the ER $\alpha$  immunostaining was mainly localized within the cytoplasm compared to controls, where the immunosignal was found only in the nuclei (Lee et al. 2004). Thus, as we can see, ovariectomy intensifies deleterious effect of aging and 17 $\beta$ -estradiol treatment counteracts it. This could explain the fact that the cytoplasmatic isoform appeared in ovariectomized animals at earlier stages, which are physiologically more damaged because of the lack of gonadal hormones, while in estradiol-treated animals, the extranuclear immunoreactivity is not noticeable until 18 months of treatment. Regarding the levels of ER67 and ER47, 17 $\beta$ -estradiol treatment is capable of reverting in part the deleterious effects of the ovariectomy in the two encephalic areas studied. The treatment induce a significant increase in ER67 and ER47 levels, compared to ovariectomized animals, which fits with Stirone's observations (Stirone et al. 2003). Our immunohistochemistry study reveals that the 17 $\beta$ -estradiol treatment, leads to an increase in total ER $\alpha$ , with the same ratio of nuclear and extranuclear isoforms as in ovariectomized animals. Although according to bibliography we could think that 17 $\beta$ -estradiol would repress the expression of its own receptor, what is going on may be a double hormone-mediated effect, regulating ER $\alpha$  expression

in a dose and age-dependent manner (Funabashi et al. 2000). Therefore, the absolute level of extranuclear ER $\alpha$  is higher when the hormone is administrated. In this sense, non-nuclear estrogen receptor has been widely described as responsible of rapid non-genomic estrogens actions (McCarthy 2008; Vasudevan and Pfaff 2008); raising the possibility that estradiol could play a neuroprotective role through a non-genomic rapid mechanism (Singer et al. 1999; Wilson et al. 2002). The way of its participation, among many others, could be in the increase of eNOS activity in cerebral blood vessels (McEwen 2001; Milner et al. 2001; Toran-Aller et al. 2002; Li et al. 2003) or the downregulation of GSK3 $\beta$  (Glycogen Synthase Kinase 3 $\beta$ ). The upregulation of this enzyme is responsible of the hyperphosphorylation of Tau protein, the main component of Alzheimer's disease amyloid (Goodenough et al. 2003).

In the diencephalon, the change in ER $\alpha$  distribution in aging is not observed, but the immunostaining remains in the nuclei through the experiment in most cases, supporting some previous works (McEwen and Alves 1999; Ishunina et al. 2000; Adams et al. 2002). As it occurs in the telencephalon, ovariectomy makes the immunostaining decrease in an age-related way. We have even observed that hormonal treatment favors the increase in both ER $\alpha$  isoforms from 12 months of treatment, in contrast to the decrease overcome by the other groups, 17 $\beta$ -estradiol treatment exerts its function through the increase in ER $\alpha$  levels, verifying the positive effect of this sexual hormone on the estradiol signaling pathway (Alonso et al. 2008). Moreover, in the diencephalon, 17 $\beta$ -estradiol exposure mitigates the loss of ER67 due to aging, so the level of the protein is higher in the estradiol-treated animals than in normal aging. None of the studied areas showed a significant presence of non-nuclear ER $\alpha$ , with the exception of some giant neurons from the supraoptic nucleus exhibiting some extranuclear labeling in the darkest immunopositive cells. The ER $\alpha$  localization within the cell in the diencephalon, almost exclusive in the nuclei, reveals other mechanisms of estrogen-mediated neuroprotection in this encephalic region different from those of the telencephalon, pointing out a specific response of telencephalon and diencephalon to aging and to estradiol treatment (Alonso et al. 2008). Regarding this, the hypothalamic nuclei are very important in the maintenance of the hypophysary–hypothalamic axis, so a rigid regulation system is needed to ensure its optimum physiological



functioning, so aging does not seem to affect the hypothalamus significantly. Thus, it has been described the lack of morphological changes in hypothalamic nuclei in relation with aging (Navarro et al. 1997). The exception to this general description is the thalamus where there is an intense extranuclear immunolabeling especially in ovariectomized animals since early age. A possible explanation to this phenomenon could be that, as we have previously attributed to extranuclear isoform of the receptor a crucial role in estrogen neuroprotective actions, this area might be especially sensitive to lack of estrogens, or the neuroprotective mechanisms were exclusively activated by non-nuclear estrogens receptor, which could be activated by autocrine extragonadal estrogens (Simpson 2003). However, we do not have any references that support this idea with reliability.

Another striking feature in both studied areas is that, with aging, the ER $\alpha$  labeling turns to acquire a heterogeneous distribution. That is, while in young animals the immunopositive neurons have a similar staining intensity as well as a uniform immunostaining distribution in the whole nucleus, aging involves huge differences in staining darkness among the immunopositive cells. Ovariectomy seems to intensify the differences in immunopositivity signal among the neurons. Related with these observations, it is possible that the extranuclear ER $\alpha$  level has a relationship with neuroprotective alternative non-genomic estrogen-induced mechanisms, according to the research works of some groups (Marin et al. 2005; Marin et al. 2003; Alonso et al. 2008). There is quite likelihood that neurons highly immunostained for ER $\alpha$  were less susceptible to neuronal death than those who lacked the receptor, being more protected by the sexual hormone, though it would be necessary the performance of more studies.

As well as this, there are some scattered little immunopositive nuclei in some telencephalic areas, which seem to belong to glial cells due to its size and localization, pointing out an involvement of glia in estrogen-mediated neuroprotection. It has been described that the presence of ER $\alpha$  in astrocytes, which could mediate indirect neuroprotection of ER-negative neurons, acting as an intermediate (Azcoitia et al. 2001a; Milner et al. 2001; Dhandapani and Brann 2007). From our results, we think that rapid mechanisms of action, activated by estrogens binding to it extranuclear receptor, could be a central point in the

activation of the neuroprotective estrogen effects. Thus, in those animals lacking gonadal hormones from youth, extragonadal estrogens could activate the estrogens-mediated neuroprotective mechanisms prematurely (Simpson 2003), which would be reflected in a higher extranuclear ER $\alpha$  levels than in control animals at the same age. Among others, the group of Azcoitia and collaborators has demonstrated the importance of brain aromatase in estrogen-mediated neuroprotection (Azcoitia et al. 2001b). Long-term exposure to 17 $\beta$ -estradiol entails a constant contribution of this sexual hormone through all the life. According to our previous results (Alonso et al. 2008), at late stages when the animals are aged, 17 $\beta$ -estradiol in E group remain stable, so the effectiveness in neuroprotection onset is better than in normal aging against age-induced stress. That is, taking our hypothesis as a starting point, delaying the change of ER $\alpha$  localization from nucleus to extranuclear sites could be a consequence of a lesser degree of neuronal stress in animals chronically exposed to 17 $\beta$ -estradiol.

Another possibility is that the key of neuroprotective effects of estradiol is related to the ER67/ER47 ratio, which is supported by Flouriot et al. (Flouriot et al. 2000). Our previous results showed that this ratio depends on the region of the brain that is studied. Regarding our results, in the telencephalon, there is a ratio ER67/ER47 in ovariectomized animals significantly higher than in the rest of the groups all throughout the study. However, the value experiments a decrease in normal aged and in ovariectomized animals from 12 months of treatment (Alonso et al. 2008). In this sense, in cell cultures it has been described as an ER67/ER47 ratio lower in proliferating cells than in quiescent or non-proliferating cultures, so when the culture is young and healthy the value of the ratio is low (Flouriot et al. 2000). There is a strong possibility that a compensatory mechanism was activated by the low levels of estrogen; 17 $\beta$ -estradiol treatment induces a progressive increase of this parameter from the beginning to the end of the experimental period, though the value never reaches up to those of the OVX group. The E group ratio, similar to that which belongs to normal-aged animals, verifies that long-term 17 $\beta$ -estradiol exposure eases ovariectomy effects by the modulation of ER67/ER47 ratio, but not those aging-associated (Alonso et al. 2008). Not only ER47 is a powerful inhibitor of ER67 AF-1 transactivation activity, but this N-terminal truncated

isoform seems to have a stronger binding affinity for the estrogen responsive element (ERE) in DNA than in ER67 (Xing et al. 1995).

Several evidences have suggested that the neuroprotective effects of estrogen may be mediated by the activation of phosphatidylinositol-3 kinase (PI3K) signaling pathways and that ER $\alpha$  but not ER $\beta$  is involved of this intracellular signaling mechanism (Cardona-Gomez et al. 2002a, 2002b; Mendez et al. 2003; D'Astous et al. 2006). The odds are that non-nuclear ER $\alpha$  is the isoform responsible of downstream activation of PI3K signaling pathway (Gundlach et al. 2000). Further investigations point ER47 to have a crucial role in mediating eNOS activation specifically (Li et al. 2003). Taking into account these observations, the higher proportion of ER47 in estradiol-treated animals at the end of the study might be favoring PI3K signaling pathway activation, being therefore a hallmark of 17 $\beta$ -estradiol-mediated neuroprotection effectiveness.

In the diencephalon, 17 $\beta$ -estradiol treatment tends to increase the ER67/ER47 ratio, even over those of the normal aged animals. Three hypotheses can explain these results: firstly, in this encephalic area, estradiol is hardly neuroprotective; secondly, the action and regulation of estrogen-dependent mechanisms is region-specific and finally yet importantly, the estrogen-mediated neuroprotection is performed in other different points of the signaling pathway. A vast majority of immunohistochemical labeling is concentrated in cellular nuclei. To the best of our knowledge, many authors support the idea that extranuclear ER $\alpha$  is responsible for most of the neuroprotective actions of estrogens, which initially do not involve gene transcription (Pappas et al. 1995; Marin et al. 2005). Moreover, during artificially induced senescence of PC12 pheochromocytoma cells, the subcellular localization of ER $\alpha$ , most of immunoreactivity was dramatically shifted from the nucleus into the cytoplasmic compartment (Lee et al. 2004). While artificial senescence is not identical to the physiological aging process, normal diploid cells in culture may achieve a non-dividing state termed "senescence" after they divide a certain amount of times, concomitant with culture aging, being a stressing state similar to physiological aging. In these situations, estrogens must activate its neuroprotective mechanisms, among which, promoting non-nuclear receptor-mediated rapid signaling pathways activation. Since a low ratio ER67/ER47 could be associated with a better functioning of short-term estradiol-

mediated neuroprotective mechanisms, the fact that in the diencephalon the ratio value was the highest, as well as a predominant nuclear immunostaining could indicate that in the diencephalic area 17 $\beta$ -estradiol treatment was not enough to restore the loss of neuroprotection effectiveness. Thus, according to our results and previous works (Alonso et al. 2008), estradiol-mediated effects are mostly exerted in the telencephalon where 17 $\beta$ -estradiol is capable of downregulating ER67/ER47 ratio, with a presence of extranuclear receptor more frequent than in normal aging animals. The diencephalon is an encephalic region that carries out very important homeostatic functions, such as the regulation of the hypothalamic–hypophysial axis, needing reliable previous neuroprotective mechanisms, so neuroprotection could be exerted by other pathways different from estrogen-activated systems.

Although none of the hypotheses expressed in this discussion can be completely discarded, our results support the idea of the existence of region-specific estrogen-mediated neuroprotective mechanisms, above all exerted in the telencephalon rather in the diencephalon in the case of non-genomic pathways. Hence, a previous work of our group (Alonso et al. 2008) demonstrates that chronic estradiol treatment improves brain homeostasis during aging in female rats through the activation of the PI3K signaling pathway in both telencephalon and diencephalon. In summary, our findings indicate that estradiol treatment during aging in ovariectomized rats may ease ovariectomy effect on ER $\alpha$  levels. As well as this, during aging and with 17 $\beta$ -estradiol treatment there is an increase in ER $\alpha$  immunoreactivity, with a global decrease in total immunolabeling in ovariectomized animals and, on the contrary, with an increase in estradiol-treated animals. As well as this, long-term treatment with 17 $\beta$ -estradiol seems to regulate ER $\alpha$  in a region-specific manner. Our findings are a starting point to look through the relationship between subcellular localization of ER $\alpha$  and the specific function in each region of the brain.

Our immunohistochemical results agree with Western blot studies in a vast majority; however, there are some faint differences between them. We cannot forget that immunohistochemistry does not allow to discern between ER67 and ER47 isoforms, and Toran-Aller and colleges have identified a novel plasma membrane-associated putative ER in neurons, called ER-X, different from classical estrogen receptors  $\alpha$  and  $\beta$  (Toran-Aller et al. 2002). The new isoform, whose apparent molecular weight is of 62–63 kDa,

reacts with antibodies to ER $\alpha$  LBD, such as our MC-20 from Santa Cruz. Moreover, immunohistochemistry and Western blot are different scientific techniques that require a differential tissue processing procedure. While for the molecular assays, the samples are stored in liquid N in the aim to guarantee the optimum conservation, the p-formaldehyde fixation before immunohistochemistry induces a modification of the three-dimensional protein conformation, with the possibility of ER-X LBD region could be accessible to the antibody and therefore recognized by immunohistochemistry but not by Western blot. In consequence, in Western blot, we are maybe underestimating total ER level, or it is possible that the 62- and 67-KDa bands were covered up. As well as this, this alternative ER-X might be mostly expressed in some exceptional situations, such as ovariectomy, which would justify the high levels of ER $\alpha$  detected in the OVX group by immunohistochemistry. Nevertheless, whatever technique was used, as a direct consequence of ovariectomy, there is a decrease in ER $\alpha$ , intensifying the aging-associated loss of protein (Adams et al. 2002; Mehra et al. 2005), while 17 $\beta$ -estradiol treatment is capable of restoring the effect of ovariectomy at later stages of the experiment.

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