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# Changes in estrogenic regulation of estrogen receptor α mRNA and progesterone receptor mRNA in the female rat hypothalamus during aging: an in situ hybridization study

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**Abstract**

We examined two molecular responses to estrogen, reduction in estrogen receptor α (ERα) mRNA and increase in progesterone receptor (PR) mRNA, in the hypothalamus of 3- (young) and 10-month-old (middle-aged) cycling, and 15-month-old (old) acyclic, Fischer 344 female rats. The rats were ovariectomized and then given silastic capsules containing 5% 17β-estradiol, or empty implants, and killed 4 days after implantation. By means of in situ hybridization, we found that, in young rats, estrogen reduced ERα mRNA in both the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) but not in the preoptic area (POA). In contrast, the effect of estrogen on ERα mRNA in the VMH and ARC of middle-aged and old rats was not statistically significant. On the other hand in all regions the induction of PR mRNA by estrogen was at least as strong in middle-aged and old as in young rats. The present study revealed that the induction of PR mRNA by estrogen in the hypothalamus was not impaired with age but ERα mRNA in the VMH and ARC was significantly impaired with age, but not in the POA. 

*Keywords:* Estrogen; Estrogen receptor α mRNA; Progesterone receptor mRNA; Aging; Ventromedial hypothalamus; Preoptic area; In situ hybridization; Senescence

## 1. Introduction

The age-related loss in estrous cycles in female rodents is associated with neuroendocrine impairment indicated by impairment in the ability to support estrous cycles with young ovarian grafts (Mobbs et al., 1984a,b) and attenuation of the luteinizing hormone surge (Lu et al., 1981; Wise, 1984a; Akema et al., 1985). Numerous studies have reported age-correlated impairment in estrogen receptors (ER) in the mediobasal hypothalamus (MBH) and preoptic area (POA) (Jiang and Peng, 1981; Blaha and Lamperti, 1983; Wise and Parsons, 1984b; Brown et al., 1990). The decrease in ER occurs early and is specific: by 8–10 months cytoplasmic ER are decreased in the MBH, including the ventromedial hypothalamus (VMH) and the arcuate hypothalamic nucleus (ARC), and the POA, but not in other brain areas or the pituitary (Wise and Parsons, 1984b; Brown, et al., 1990).

On the other hand, the induction of hypothalamic progesterone receptors (PR) by estrogen is somewhat impaired during reproductive senescence as well as ER (Wise and Parsons, 1984b), although it is not known if the lower levels of measurable PRs is due to lower levels of PR mRNA, lower translation, or impairments in the post-translational processing of the protein.

These and similar reports have stimulated the hypothesis that reproductive senescence in female rodents

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is due, at least in part, to a relative insensitivity to estrogen in the hypothalamus, but it has been shown that the induction of neurotensin mRNA by estrogen is not impaired with age (Miller et al., 1994; Funabashi et al., 1998). These reports suggest that, at least as reflected by the mRNA level, reproductive senescence in rats is not due to a general decrease in sensitivity to estrogen. Indeed, there is evidence of an increased responsiveness to estrogen with age (Mobbs, 1994).

In the present study, therefore, we examined two molecular responses to estrogen, a reduction in ERα mRNA and an increase in PR mRNA in the hypothalamus during reproductive senescence. In particular, an increase in PR mRNA caused by estrogen is a good marker when looking for changes in estrogen regulation during aging, since it is well established that estrogen greatly increases PR mRNA in the hypothalamus of young rats (Romano et al., 1989; Simerly et al., 1996; Shughrue et al., 1997).

## 2. Materials and methods

### 2.1. Animals

Fischer 344 female rats, 2 (young, $n = 12$), 9 (middle aged, $n = 10$), and 14 months old (old, $n = 12$), were purchased from the NIA aging colony and maintained on food and water ad libitum on a 12:12 light cycle (07:00 h lights on, 19:00 h lights off). As determined by vaginal smear pattern, young and middle-aged rats exhibited regular 4-day cycles, and old rats exhibited constant diestrus. Ten days after the ovariectomy, rats were given silastic capsules containing 5% 17β-estradiol (OVX + E group) or empty implants (OVX group), and killed between 09:00 and 11:00 h 4 days after implantation under $CO_2$ anesthesia. Implant sizes were assigned according to body weight to provide the same implant length/g body weight (1 mm/40 g) for different ages. In fact, serum concentrations of estradiol were identical in all groups (Funabashi et al., 1998).

### 2.2. In situ hybridization

In situ hybridization was carried out as described previously (Funabashi et al., 1995). Briefly, frozen 10 μm thick sections were fixed in 3% paraformaldehyde in 0.1 M phosphate buffer, dehydrated in ethanol, and stored at −20°C until use. Sections from various rats were matched according to the anterior to posterior level by examining cresyl-violet-stained sections which were taken every 200 μm during sectioning of brains. Around 2 h after prehybridization, hybridization was carried out in the same buffer containing 10% dextran sulfate, and labeled probe ($1 \times 10^6$ cpm/35 μl/section) overnight at 42°C. Slides were dipped in 2 × SSC, then washed twice in 1 × SSC, followed by incubation overnight in 4 l of 0.1 × SSC at room temperature with gentle stirring. On the next day, the slides were washed in 0.1 × SSC at 42°C for 1 h. Then sections were dehydrated in ethanol, air-dried and then apposed to X-ray film for 3 days. The signals were determined by means of a computerized densitometric scanning (MCID system, Ontario, Canada). We analyzed the background level of each section and did not find any difference among groups in the background. The region of interest was outlined bilaterally on adjacent sections and two measures were obtained; Ar, area of the region to be measured and ROD, relative OD within that region. We then took mRNA levels as the index of (ROD signal−ROD background) × area. This procedure is the same as previously reported (Funabashi et al., 1995) and we confirmed that the index obtained has a close relation to levels of mRNA expression in northern blot. The index of mRNA levels relative to the value in young OVX rats was arbitrarily set at 100%. Comparisons of the effects of age and estrogen were carried out by analysis of variance followed by Fisher's protected LSD post-hoc test, and significance was attained at $P < 0.05$ (Fig. 4).

### 2.3. Probes

Probes for detection of ERα mRNA and PR mRNA were internally labeled with $^{32}$P-dCTP (800 Ci/mmol; NEN Research Products) by amplified primer extension labeling to produce a single-stranded DNA probe as reported previously (Funabashi et al., 1995). ERα cDNA was obtained by reverse transcription-polymerase chain reaction (N′-gAAggCATggTggAgATCTTT; C′-TgTTgTAgAgATgCTCCATgC) and confirmed as a part of rat ERα cDNA (Koike et al., 1987) by direct sequence. Rat PR cDNA corresponding to the steroid binding domain was generously donated by Dr Junzo Kato (Hagihara et al., 1992). Since this region of the rat PR cDNA is common to the rat PR forms A and B (Kato et al., 1994), PR mRNA levels described in the present study represent the sum of PR forms A and B mRNA levels.

## 3. 3. Results

### 3.1. Changes in ERα mRNA in the hypothalamus

As seen in Figs. 1 and 3, the distribution of intense signals for ERα mRNA and PR mRNA was identical to those reported previously (Lauber et al., 1990; Simerly et al., 1990; Hagihara et al., 1992). As a control, no intense signal was detectable with the sense probe (data not shown). We also confirmed the specificity of the probe by northern blot (data not shown).

ANOVA revealed a significant effect of age on the ERα mRNA level in the POA of OVX rats ($P = 0.024$) but not of OVX + E rats. Post-hoc comparison revealed that the ERα mRNA level in middle-aged OVX rats was significantly higher than that in young OVX rats ($P = 0.05$). Estrogen did not significantly change ERα mRNA levels in either young or old rats.

In the VMH, ANOVA revealed the significant effect of age on the ERα mRNA level in OVX rats but not in OVX + E rats. Post-hoc comparison revealed that ERα mRNA level in middle-aged OVX was significantly lower than that in young OVX rats ($P = 0.041$) and old OVX rats ($P = 0.0058$). Estrogen treatment significantly reduced the ERα mRNA levels in young OVX rats ($P < 0.05$), but in middle-aged and old rats, estrogen did not have a statistically significant effect. Alternatively, ERα mRNA level in the VMH of middle-aged OVX rats was significantly lower than that of young OVX rats, so that the ERα mRNA level in middle-aged OVX + E rats was the same as that in young OVX + E rats. Therefore, estrogen might fail to decrease the ERα mRNA level in middle-aged rats.

In the ARC, estrogen decreased ERα mRNA in young rats, but did neither in middle-aged nor old rats. ANOVA revealed no significant effect of age on the ERα mRNA level in OVX and OVX + E rats.

### 3.2. Changes in PR mRNA in the hypothalamus

In the POA, estrogen significantly increased the level of PR mRNA in all aged groups. ANOVA revealed no significant effect of age on PR mRNA in OVX and OVX + E rats.

In the VMH, estrogen significantly increased the level of PR mRNA regardless of age as well as in the POA. In addition, although it was not found statistically significant by ANOVA, the level of PR mRNA in estrogen-implanted rats was increased with age, but was not in the POA.

In the ARC, again, estrogen significantly increased the level of PR mRNA regardless of age, as well as that in the VMH and the POA.

## 4. Discussion

In agreement with previous reports on studies by means of in situ hybridization (Lauber et al., 1990; Simerly and Young, 1991; Miller et al., 1994) and northern blot (Funabashi and Kimura, 1994), estrogen reduced ERα mRNA in the MBH of young rats, including the VMH and the ARC, but we confirmed the finding in our previous report that estrogen did not decrease ERα mRNA in the POA of young rats as it

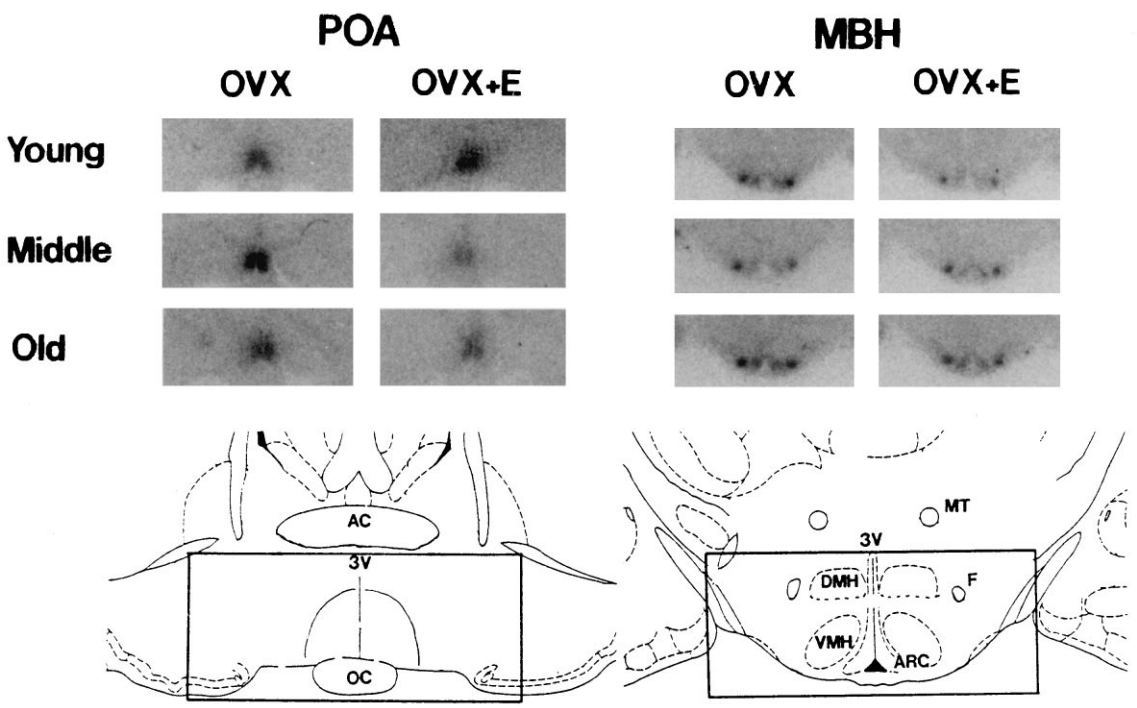


Fig. 1. Representative X-ray film autoradiographs of sections through the rat hypothalamus hybridized with $^{32}$P-labeled ERα cDNA probes. Schematic illustrations of coronal sections, corresponding to X-ray films, are shown in the lower column and the bold line indicates the brain shown in X-ray films. Strong hybridization signals were only seen in the periventricular nucleus of the POA (left side) and VMH pars ventrolateralis and ARC (right side). AC, anterior commissure; OC, optic chiasm; 3V, third ventricle; DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; ARC, arcuate hypothalamic nucleus; MT, mammillothalamic tract; F, fornix.

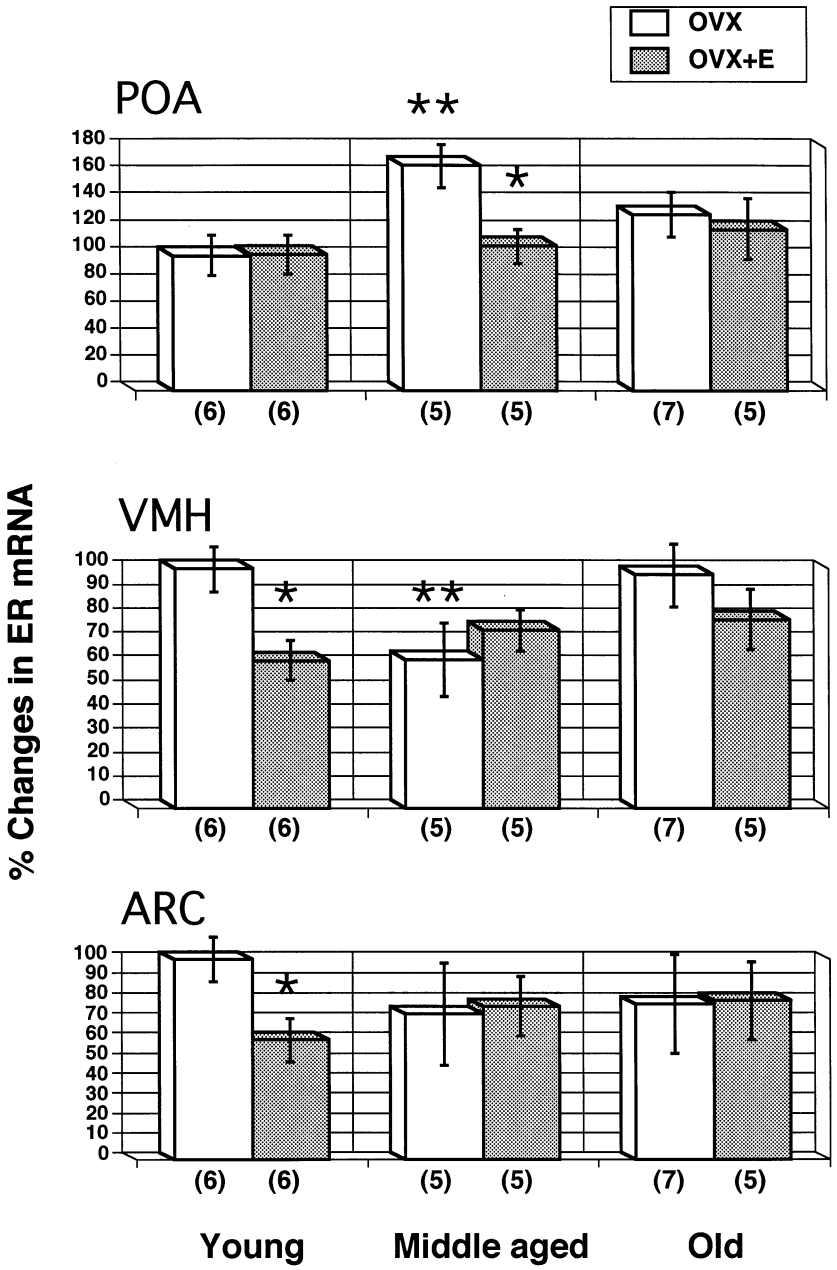


Fig. 2. Quantitation of ERα mRNA levels in the female rat POA and MBH expressed as percentage of young OVX mean values. $*P < 0.05$ vs. OVX and $**P < 0.05$ vs. young OVX rats by ANOVA followed by post-hoc test. Each column and vertical line indicates the mean and SE, respectively. Numbers in the parentheses indicate numbers of rats.

did in the MBH (Funabashi and Kimura, 1994). Therefore, this indicates that estrogenic regulation of ERα mRNA in young rats is different in the MBH from that in the POA, as previously suggested (Shughrue et al., 1992; Funabashi and Kimura, 1994). In line with this, it has been shown that estrogen decreases staining intensity for ERα in the VMH but not in the POA by means of immunocytochemistry (Don Carlos et al., 1995). In addition, it has been shown that estrogenic regulation of ERα is different in the POA from that in the MBH during development (Orikasa et al., 1996). Although contradictory effects of estrogen on the immunoreactivity of ERα (Yuri and Kawata, 1991) and the expression of ERα mRNA (Miller et al., 1994; Simerly et al., 1996) in the POA are reported, the difference between our results and theirs could be due to the difference in duration and/or the dose of estrogen.

Nevertheless, even by middle age before the estrous cycle ceases, ERα mRNA in the MBH was not significantly reduced by estrogen. Again, this result is also in good agreement with our previous report on northern blot (Funabashi and Kimura, 1994). In the MBH, ERα

mRNA in middle-aged OVX rats was lower than that in young OVX rats, and was in fact not statistically different from the level in estrogen-implanted young rats. Therefore, even if estrogen were inhibiting ERα mRNA in the middle-aged rats to the same degree as in young estrogen-implanted rats, the effect of estrogen would not be statistically significant. The present data cannot therefore distinguish between an impaired responsiveness of ERα mRNA to estrogen and a floor effect due to an age-correlated decrease in ERα mRNA. In old rats as in middle-aged rats, the effect of estrogen on ERα mRNA in the MBH was not statistically significant.

In the POA, ERα mRNA in middle-aged OVX rats was significantly higher than that in young rats. Although we do not have any immediate explanation of this finding, the data are consistent with the results obtained by northern blot (Funabashi and Kimura, 1994) even though we used a different strain, estrogen treatment and method, it could not be due to a technical problem. We speculate that this high expression of ERα mRNA in middle-aged OVX rats is related to impairment of the estrous cycle which will be followed. In addition, the arginine-vasopressin receptor mRNA in the POA of middle-aged OVX rats is also increased as compared to that of young OVX rats (Funabashi et al., 2000b).

In contrast, the induction of PR mRNA by estrogen was not impaired during aging, even in old rats. In fact, the induction of PR mRNA by estrogen increased monotonically with age in the MBH, although the differences among the ages were not statistically significant. Interestingly, in the POA, there was no remarkable difference in the induction of PR mRNA by estrogen with age, whereas PR mRNA in OVX rats was not significantly increased with age. We further observed a greater effect of estrogen on PR mRNA in the ARC than in the VMH. These results may indicate a different responsiveness to estrogen or possibly different rates of mRNA degradation in those regions. The unimpaired induction of PR mRNA by estrogen during aging is consistent with the study of Brown et al. (1990), which measured the induction of progesterone receptors by ligand binding.

We have also found that induction by estrogen of neurotensin mRNA (Funabashi et al., 1998), which is in good agreement with the result reported by Miller et al. (1994), and of preproenkephalin mRNA (Funabashi et al., unpublished observation), oxytocin receptors and prolactin (Mobbs et al., unpublished observation) is not impaired during female rat reproductive senescence. On the other hand, we found, as described above, that inhibition of ERα mRNA, like inhibition of preproopiomelanocorticotropin mRNA (Lloyd et al., 1991; Karelus and Nelson, 1992) is attenuated during aging. Therefore, the pattern which is beginning to emerge is that the response of estrogen-stimulated parameters is not impaired with age, but the response of estrogen-inhibited parameters becomes less demonstrable during aging, a pattern possibly (though speculatively) related to persistent effects of estrogen which accumulate during aging (Mobbs, 1994). In any case, it seems clear that refractoriness to estrogen is not a general characteristic of reproductive senescence. Interestingly, cellular responsiveness during aging is not always decreased, but certain responses of cells, in particular toxic response, is increased with age (see reviews by Rattan and Derventzi, 1991 for details). However, it seems less likely that the dramatic age-correlated impairments in the estrogen-stimulated LH surge are due to insensitivity to estrogen per se (in the static sense), and more likely that these impairments are due to age-related changes in dynamic properties, including possible changes in translocation of estrogen receptor kinetics (Wise and Parsons, 1984b) or, even more likely, age-correlated changes in properties of the endogenously-

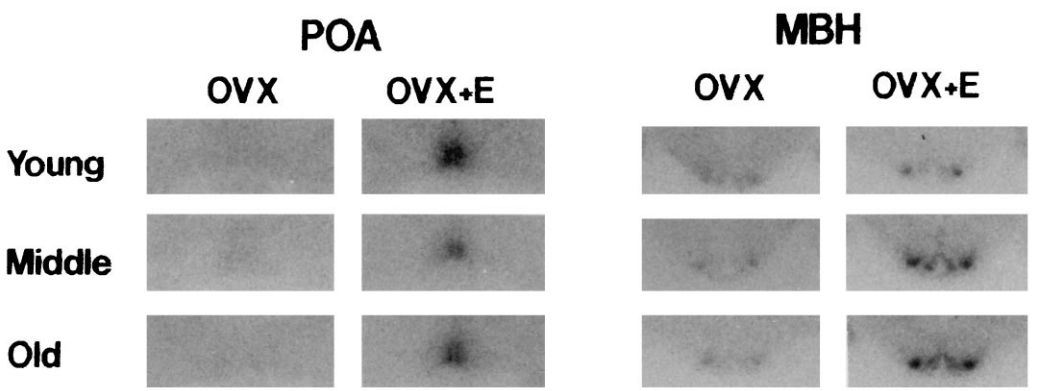


Fig. 3. Representative X-ray film autoradiographs of sections through the rat hypothalamus hybridized with $^{32}$P-labeled PR cDNA probes. In the POA of OVX + E rats, intense signals were only seen in the periventricular nucleus of the POA. In the mediobasal hypothalamus of OVX + E rats, intense signals were observed in the VMH pars ventrolateralis and ARC. Note: very faint signals were seen in OVX rats. For further details, see Fig. 1.

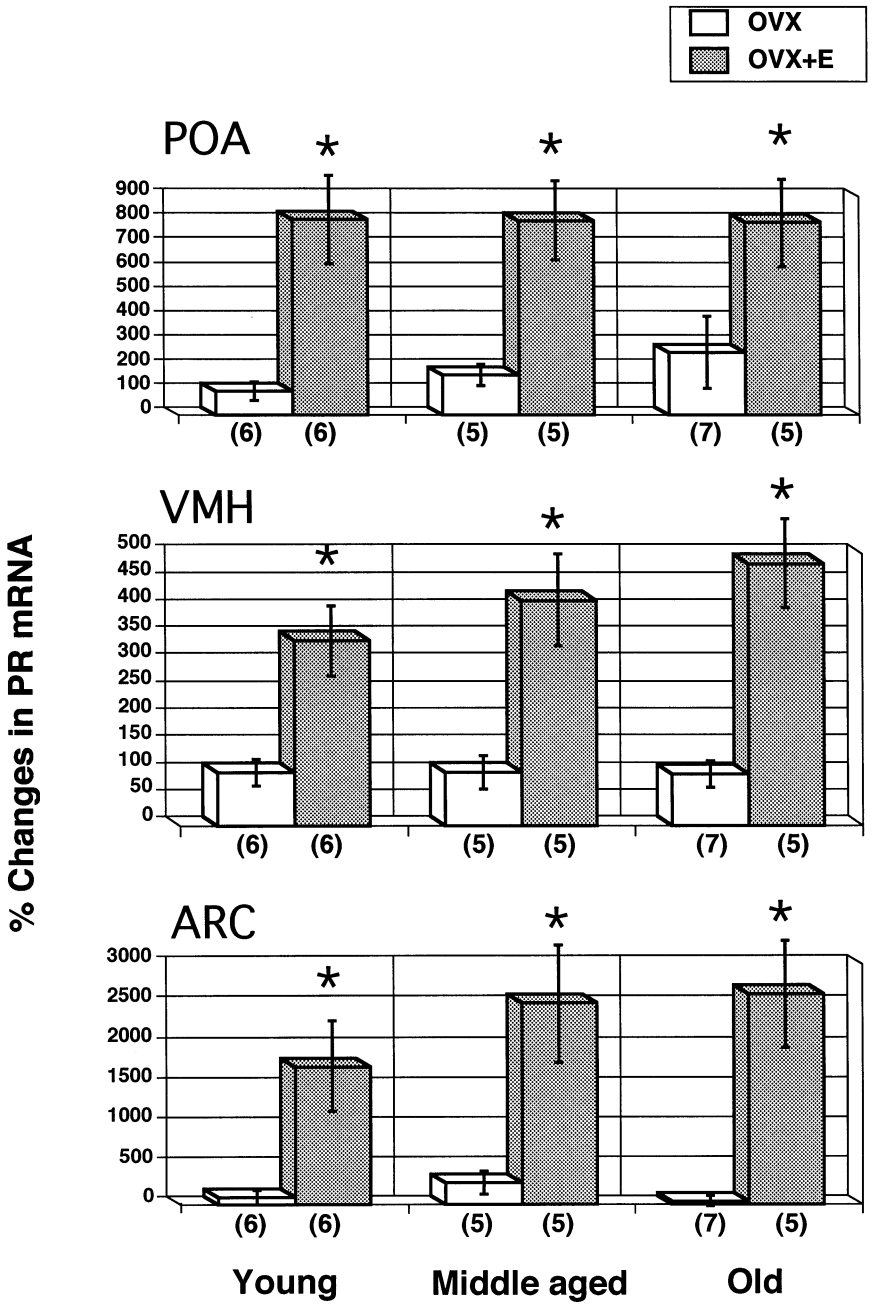


Fig. 4. Quantitation of PR mRNA levels in the female rat POA and MBH expressed as percentages of young OVX mean values. *$P < 0.05$ vs. OVX by ANOVA followed by post-hoc test. For further details, see Fig. 2.

generated rhythms (Wise et al., 1997). In support of this view, we recently reported that age-related impairments in the luteinizing hormone surge are possibly due to impairments in the regulation of arginine-vasopressin receptor post-synaptic to AVP neurons (Funabashi et al., 2000b) which convey the clock information to GnRH neurons (Funabashi et al., 1999; Palm et al., 1999; Funabashi et al., 2000a).

In summary, the present study shows that estrogenic reduction of ERα mRNA in the MBH, but not in the POA, is impaired with age, but estrogen is still capable of inducing PR mRNA in the MBH and the POA. Furthermore, estrogenic regulation of ERα mRNA is different in the POA and the MBH, and the magnitude of induction of PR mRNA by estrogen is different in those hypothalamic areas. The latter finding may be associated with the estrogen-related functional difference between the POA and MBH. Whether the changes observed in the mRNA level reflect changes in the transcriptional rate, mRNA stabilization, or translation to proteins, remains to be determined, since at least in the case of ER, it is suggested that ER protein levels in

the POA are controlled by cellular posttranscriptional mechanisms (Pasterkamp et al., 1997). In addition, it is interesting to investigate whether ERβ is involved in reproductive senescence, since it has been indicated that the role of ERβ in reproductive functions is different from that of ERα (Couse and Korach, 1999).

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