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Mechanisms of Ageing and Development
123 (2002) 593–601

mechanisms of ageing and development

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Age differentially influences estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) gene expression in specific regions of the rat brain

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Received 3 August 2001; received in revised form 19 October 2001; accepted 19 October 2001

Abstract

Estradiol's ability to influence neurochemical events that are critical to female reproductive cyclicity and behavior decreases with age. We tested the hypothesis that decreases in estrogen receptor-α (ERα) and/or ERβ mRNA explain the brain's declining responsiveness to estradiol. We assessed ERα and ERβ mRNA levels in intact and ovariectomized estradiol-treated rats. ERβ mRNA was detected in several brain regions and decreased by middle-age in the cerebral cortex and supraoptic nucleus of estradiol-treated rats. ERβ mRNA levels exhibited a diurnal rhythm in the suprachiasmatic nucleus of young and middle-aged rats and this rhythm was blunted in old rats. We examined ERα mRNA in the periventricular preoptic, medial preoptic, ventromedial and arcuate nuclei, and it was decreased only in the periventricular preoptic nucleus of the old rats. In summary, the expression of ERα and ERβ mRNAs is differentially modulated in the aging brain and changes are region specific.

Keywords: Estradiol; ERα; ERβ; Aging; Female

1. Introduction

During aging, the hypothalamic-pituitary-gonadal axis becomes less responsive to the feedback effects of estradiol. This loss may contribute to the transition from regular to irregular estrous cycles and ultimately to reproductive senescence. Decreasing responsiveness to estradiol may lead to the alterations in the timing and amplitude of proestrous and steroid-induced LH surges (Wise,

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0047-6374/02/$ - see front matter
PII: S0047-6374(01)00406-7

1982, 1984) and to the decreasing amplitude and frequency and increasing duration of LH pulses (Scarbrough and Wise, 1990) that have been observed in middle-aged female rats. Furthermore, the ability of steroids to induce reproductive behavior is also blunted. Aging female rats exhibit significantly less lordotic behavior in response to estradiol stimulation (Cooper and Linnoila, 1977; Gray et al., 1980; Wise and Parsons, 1984) than young animals (Wise et al., 1984). In addition, middle-aged female rats perform poorly compared to young animals in tests of cognition and memory (Markowska, 2000). Importantly, these changes become apparent in middle-aged (8–12 months) rats, prior to detectable changes in circulating estradiol levels (Lu, 1983), suggesting that decreasing responsiveness to estradiol may lead to a declining ability to influence reproductive and non-reproductive actions in the brain.

It is generally accepted that to date, most of the physiologically important actions of estrogen are mediated by nuclear receptors that act as transcription factors following estrogen binding (Gorski et al., 1993; Glass, 1994). There are two known subtypes of the nuclear estrogen receptor: ERα and ERβ. Both receptors bind estrogen with a high affinity and bind to the same DNA response element. Significant differences exist in the area of the protein responsible for interactions with other transcriptional machinery, suggesting different mechanisms of transcriptional activation (Kuiper et al., 1998). The mRNAs for each receptor subtype have been thoroughly mapped in the rat brain (Shughrue et al., 1997). In general, ERα is highly expressed in the anteroventral periventricular, medial preoptic, arcuate, and ventromedial nuclei and the amygdala; areas of the brain that are responsible for reproductive functions and behaviors. Whereas, ERβ mRNA is concentrated in the cerebral cortex, hippocampus, periventricular preoptic, preoptic, bed nucleus of the stria terminalis, paraventricular and supraoptic nuclei, amygdala and other brain regions that indicate non-reproductive actions of estradiol. However, ERβ is expressed in some regions that are involved in reproduction, such as the periventricular preoptic and medial preoptic nuclei. Interestingly, it has been recently reported that ERβ mRNA and protein, but not ERα, is expressed in GnRH neurons (Hrabovszky et al., 2000), opening the possibility that ERβ may directly influence GnRH synthesis and secretion. There are also several regions of the brain which express both receptors (Shughrue et al., 1998). It is hypothesized that interactions between the two receptors may take place in these brain regions to mediate estradiol's actions.

It has previously been hypothesized that diminished responsiveness to estradiol may be mediated by a decrease in the density of estrogen receptors. While under certain physiological conditions, changes in estradiol binding have been observed (Wise et al., 1984; Wise and Parsons, 1984; Wise and Camp, 1984), no changes in ERα mRNA levels have been detected (Miller et al., 1994; Funabashi et al., 2000). The expression of mRNA for the recently discovered ERβ subtype, however, has not been examined in aging animals. Therefore, we focused on the analysis of age-related changes in the expression of ERβ mRNA in various brain regions including regions of the brain that are thought to mediate the reproductive as well as non-reproductive actions of estrogen. We also examined levels of ERα mRNA expression throughout aging for comparison.

2. Methods

2.1. Animals

Female Sprague–Dawley rats were maintained in the University of Kentucky AALAC-approved animal facility under a 14/10 light/dark cycle (lights on at 04:00 hours) with access to food and water ad libitum. We assessed ER mRNA levels in: (1) intact and (2) ovariectomized (OVX) estradiol-treated rats. This strategy was used to assess ER changes: (1) that occur with normal aging (intact animals) and (2) under experimentally controlled endocrine conditions in which we maintained equivalent levels of serum estradiol in all the age groups (OVX, estradiol-treated). The use of aging rats under endocrine controlled conditions eliminates the potential confounding effects of differing levels of ovarian hormones that ac-

company advancing stages of reproductive senescence. In the first experiment, young (3–4 months), middle-aged (11–12 months) and old (19–24 months) female rats were used intact. In a second experiment, we ovariectomized young, middle-aged and old rats for 1 week and subcutaneously implanted Silastic capsules (0.062/0.125 in. inner/outer diameter) containing estradiol dissolved in oil (180 μg/ml, 20 mm long in young rats, 30 mm long in middle-aged and old rats) for 2 days. We have reported previously that this treatment produces equivalent levels of serum estradiol in all the age groups (Wise et al., 1981). Prior to ovariectomy all the young rats exhibited at least two regular estrous cycles, all the middle-age rats exhibited irregular estrous cycles, and all the old rats were in persistent diestrus. Rats were killed by decapitation, brains removed, frozen and sectioned (12 μm thick). Since the suprachiasmatic nucleus is the major circadian neural pacemaker in the mammals, and virtually every neurochemical analyzed to date exhibits a 24 h rhythm (Inouye and Shibata, 1994; Albers et al., 1991), we examined the levels of ER mRNA in rats that were killed at seven times of day (23:00, 03:00, 08:00, 12:00, 16:00, 20:00, and 23:00 hours).

2.2. In situ hybridization

In situ hybridization was performed using previously described methods (Shughrue et al., 1997). Briefly, specific probes for ERα and ERβ were used: the ERα probe consisted of 800 nucleotides of the 5′end of the ERα cDNA; the ERβ probe was a cocktail containing a 558-bp and a 285-bp probe shown to be specific for ERβ (Shughrue et al., 1996). cRNA probes were generated by transcription in the presence of 50 μM ^{35}S-UTP and non-radioactive ATP, CTP and GTP (500 nM of each). Sections were hybridized overnight in an open air humidification chamber with a solution containing 6×10^6 dpm probe per slide, 50% formamide, 10% dextran sulfate, 1X Denhardt's solution, 0.3M NaCl, 0.2M DTT at 55 °C. Slides were washed in 2X SSC/10 mM DTT, treated with RNAse A (25 μg/ml), and washed two times in 0.1X SSC at 63 °C for ERα and 65 °C for ERβ. Following dehydration, slides were immersed in NTB2 nuclear emulsion for 30 days. Following photographic development, slides were stained with cresyl violet and coverslipped for analysis.

2.3. Quantification of mRNA expression and statistical analysis

The density of silver grains per cell was determined following development of emulsion-coated slides. BIOQUANT V4.0 was used to determine the area of grains above a user-defined threshold per labeled cell. A cell was considered labeled if the density of grains covering the cell reached five times that of a non-labeled cell in the same brain section (background).

Data were statistically analyzed by two-way analysis of variance (ANOVA, time × age) for measurements of mRNA levels in the suprachiasmatic nucleus, and by one-way ANOVA for all the other brain regions. The Student Neuman–Keuls test was used for post-hoc analysis. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. ERβ mRNA levels in the cerebral cortex of intact female rats

We initially examined ERβ mRNA levels in the intact young, middle-aged and old rats. Fig. 1 shows the mean grain area per cell of ERβ

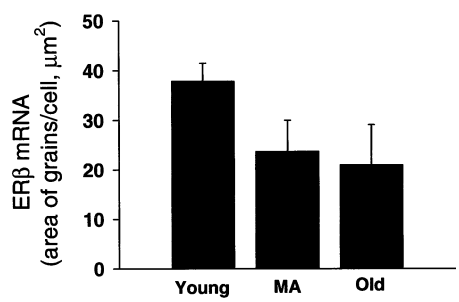

Fig. 1. ERβ mRNA levels in the cortex of aging intact rats. Bars represent the mean ± SEM, $n = 4$–5. No statistically significant differences were observed ($P = 0.158$).

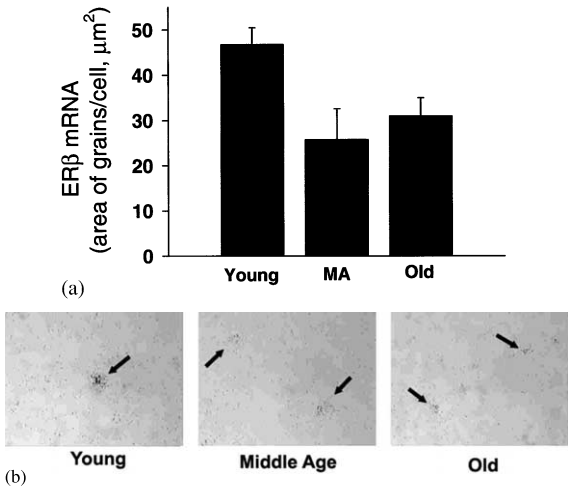

Fig. 2. (A) ERβ mRNA levels in the cortex of OVX, estradiol-treated rats during aging. There was a significant effect of age ($P < 0.02$) and ERβ mRNA levels decreased by the time rats were middle-aged ($P < 0.05$). Bars represent the mean ± SEM, $n = 4–5$. (B) Photomicrographs of ERβ in situ hybridization in young, middle-aged, and old animals. Arrows point to labeled cells. Magnification = 400 × .

mRNA in cells of layers IV–VI of the cerebral cortex (isocortex region). Due to the variation in the middle-aged and old groups, average mRNA levels exhibited a tendency to decrease, but this change did not reach statistical significance ($P = 0.158$).

3.2. ERβ mRNA levels in ovariectomized estradiol-treated rats

To control for the variable levels of serum estradiol that occur in female rats during various stages of reproductive aging (Lu, 1983), we ovariectomized all animals and implanted subcutaneous Silastic capsules that contain estradiol dissolved in sesame oil. Equivalent levels of serum estradiol are produced with this paradigm (Wise and Parsons, 1984). ERβ mRNA levels in the cortex of these animals are shown in Fig. 2A. Brains collected at 12:00 hours were analyzed since no effect of time of day was observed (data not shown). Under these controlled endocrine conditions, we detected a significant effect of age ($P < 0.02$). The mean grain area was reduced significantly by middle age ($P < 0.05$). Representative photomicrographs are shown in Fig. 2B.

ERβ mRNA levels were also examined in the suprachiasmatic nucleus (Fig. 3). This nucleus is the major circadian neural pacemaker in mammals and drives the circadian aspects of female reproductive function. Most neurochemical aspects of suprachiasmatic nucleus exhibit diurnal rhythmicity; therefore, rats were killed at seven different times during the day (23:00, 03:00, 08:00, 12:00, 16:00, 20:00, and 23:00 hours) to assess both the potential changes in rhythmicity of ER mRNA expression as well as changes in the overall average level of receptor expression. ERβ mRNA expression in the suprachiasmatic nucleus was low but detectable. Two-way ANOVA revealed a significant interaction between the age and time ($P < 0.04$). There was a significant effect of time-of-day in both the young and middle-aged rats ($P < 0.001$). Although the same trend was observed in old rats, the rhythm was not statistically significant ($P = 0.057$). The regulation of the single pulse of expression appeared to be lost as levels were also slightly elevated in the evening.

ERβ mRNA expression was also examined in the periventricular preoptic, medial preoptic, paraventricular, and supraoptic nuclei of OVX estradiol-treated rats (Fig. 4A–D). No significant changes in the expression were observed with age in the periventricular preoptic nucleus ($P = 0.241$; Fig. 4A), medial preoptic nucleus ($P = 0.460$; Fig. 4B) or paraventricular nucleus ($P = 0.601$; Fig.

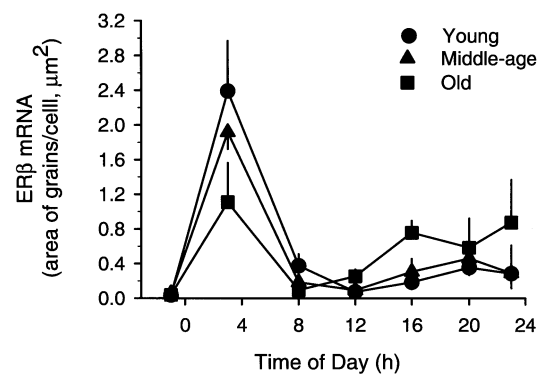

Fig. 3. ERβ mRNA levels in the suprachiasmatic nuclei at various times of day. Young and middle-aged rats exhibited a diurnal rhythm of expression peaking at 03:00 hours ($P < 0.001$). The effect of time was lost in the old animals ($P = 0.057$, $n = 5–7$ per time point for each age group).

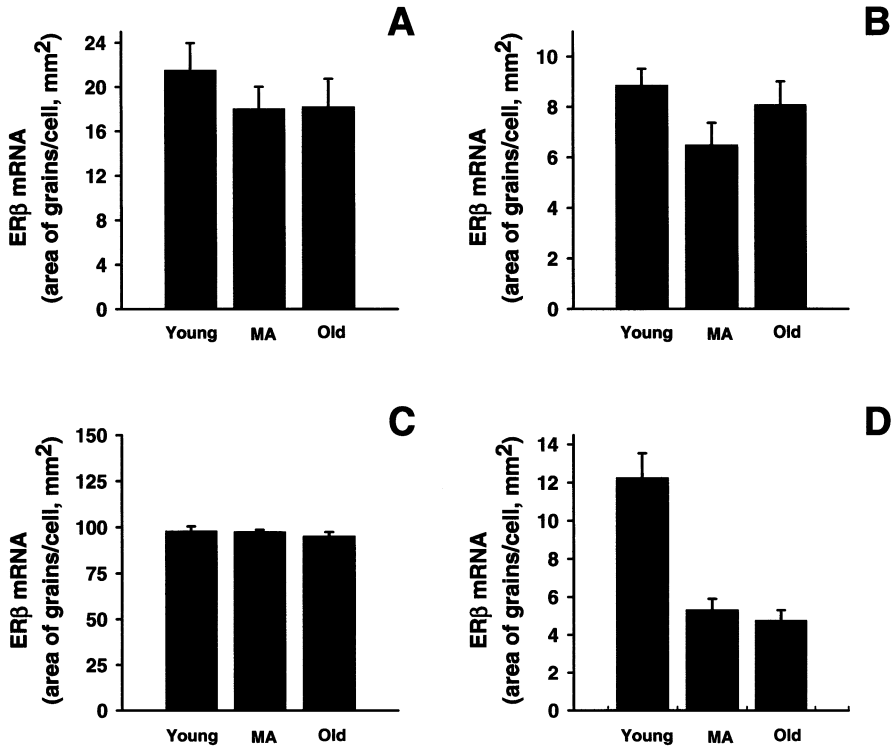

Fig. 4. ERβ mRNA levels in the: (A) periventricular preoptic nucleus; (B) medial preoptic nucleus; (C) paraventricular nucleus; and (D) supraoptic nucleus. Bars represent the mean ± SEM, $n = 4–5$. A significant decline with age was observed in the supraoptic nucleus (D) ($P < 0.05$).

4C). In contrast, ERβ mRNA expression was significantly reduced in the middle-aged and old animals in the supraoptic nucleus ($P < 0.05$; Fig. 4D).

3.3. ERα mRNA levels in ovariectomized estradiol-treated rats

For comparison, we examined ERα mRNA expression in the periventricular preoptic, medial preoptic, arcuate, and ventromedial nuclei, four regions of the hypothalamus that control reproduction (Fig. 5). ERα mRNA levels decreased in the periventricular preoptic nucleus by the time female rats were old ($P < 0.002$), but were not altered with age in the medial preoptic nucleus ($P = 0.880$), ventromedial nucleus ($P = 0.33$), or arcuate nucleus ($P = 0.405$). We did not detect ERα mRNA in any other region that we analyzed (i.e. cerebral cortex, suprachiasmatic, supraoptic, or paraventricular nuclei).

4. Discussion

In these studies, we examined the regional and age-dependent expression of ERβ and ERα mRNA in the female rat brain. Our study focused attention on ERβ since this newly discovered receptor is expressed in the brain (Shughrue et al., 1997) and may mediate different actions than the classical ERα (Nilsen et al., 2000; Patrone et al., 2000) and has never been assessed in aging animals. We report three important findings. First, we found both regional and age-related differences in ERα and ERβ mRNA expression. Second, combining animals at varying stages of reproductive senescence appears to increase the variation in ERβ mRNA levels in each age group and hide potentially important changes in the level of receptor expression. In contrast, when estradiol levels are controlled, ERβ mRNA levels in the cerebral cortex and supraoptic nucleus decreased in the middle-aged and old rats, while the

rhythm of expression in ERβ mRNA in the suprachiasmatic nucleus was absent in old animals. There was no effect of age on ERβ mRNA in the periventricular preoptic, medial preoptic or paraventricular nuclei. Third, ERα mRNA levels were relatively immune to the effects of age, exhibiting decreases only in the periventricular preoptic nucleus of the old rats.

The effects of aging on ERβ mRNA expression in the brain are not global, but instead are region-specific. In the cerebral cortex, ERβ mRNA expression decreases by middle age. It is important to point out that we were only able to detect these age-related changes when the hormonal milieu of all the age groups was controlled. In random cycling middle-aged female rats in which variable hormonal conditions accompany estrous cyclicity and reproductive aging, the variance was too great to achieve statistical significance. It is interesting to note that this decline in receptor expression correlates with the cognitive decline seen in the middle-age rats (Markowska, 2000). In addition, on proestrus, when circulating estradiol levels are highest, rats perform better on tasks that may require cortical function (Warren and Juraska, 1997). These observations in rats have recently been supported by studies in aging women (Keenan et al., 2001). In postmenopausal women estrogen replacement therapy enhances cognitive deficits that involve cortical memory.

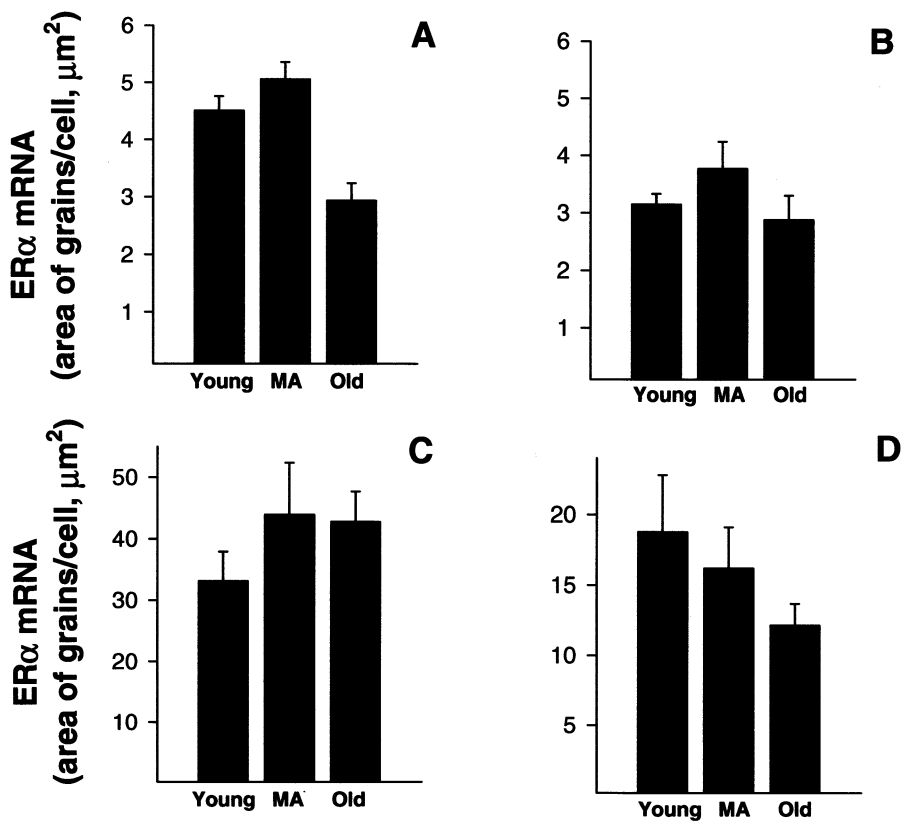

Fig. 5. ERα mRNA levels in the: (A) periventicular preoptic nucleus; (B) medial preoptic nucleus; (C) arcuate nucleus; and (D) ventromedial nucleus. Age significantly reduced mRNA in the periventricular preoptic nucleus ($P < 0.002$). The mRNA levels did not change with age in the medial preoptic nucleus ($P = 0.880$), arcuate nucleus ($P = 0.405$), or the ventromedial nucleus ($P = 0.33$). Bars represent the mean ± SEM, $n = 4–5$.

Some of the neuroprotective effects of estradiol appear to require estrogen receptors to protect the brain against neurodegenerative diseases and injury (Dubal et al., 1998; Wise et al., 2001). Which receptor subtype mediates these neuroprotective actions has been an area of active discussion. Our previous studies in mice demonstrate that ERβ does not appear to play a critical neuroprotective role against stroke-like injury, since estradiol continues to effectively protect against injury in ERβKO mice while failing to do so in ERαKO mice (Dubal et al., 2001). We have also reported that the ability of estradiol to protect does not diminish in middle-aged rats (Alkayed et al., 2000; Dubal and Wise, 2000). The present data add credence to our hypothesis that neuroprotection against ischemic injury involves ERα, but not ERβ, since the decrease in the mRNA levels of this receptor subtype in the cerebral cortex of the middle-aged rats does not appear to impact on the ability of estradiol to protect against ischemia.

In the suprachiasmatic nucleus, we detected a diurnal rhythm of ERβ mRNA levels in young and middle-aged rats and rhythmic expression was not detectable in old animals. The overall basal level of expression does not appear to be diminished, but the temporal regulation is lost. This alteration in a daily rhythm may contribute to the decreasing ability of estradiol to 'organize' daily signals from the suprachiasmatic nucleus that, in turn, regulate GnRH neurons. We have shown previously that the rhythm of norepinephrine turnover (Wise, 1984) and the density of α_1-adrenergic receptors are blunted in the suprachiasmatic nuclei of middle-age rats (Weiland and Wise, 1990). ERα is not detectable in the suprachiasmatic nucleus; thus ERβ may mediate the estrogen signal in this brain region. Our current observations of altered rhythmicity of ERβ gene expression in older animals may be important in understanding the decline of numerous estradiol-dependent circadian-driven neurochemical signals that have been reported (Wise et al., 1997).

In contrast to the age-related changes in ERβ gene expression in multiple brain regions, we found that ERα mRNA levels are largely resistant to age-dependent changes. The only change we observed was between middle-aged and old rats and was confined to the periventricular preoptic nucleus. These changes were detectable considerably later in the lifespan than the decline in hypothalamic events associated with reproductive decline (Wise et al., 1999). In the ventromedial nucleus there was a trend towards a decline in ERα expression with age, however, this did not reach statistical significance. These results confirm and extend those of Miller et al. (1994), who showed that ERα mRNA levels do not change in several areas of the hypothalamus when animals were treated with long-term estradiol replacement. However, Funabashi et al., (2000) recently showed that estradiol's ability to down-regulate ERα mRNA disappears in middle-aged and old rats. The difference in the regulation of ERα and ERβ mRNA with age warrants further investigation.

In summary, our data show that ERα and ERβ mRNA are expressed in several regions of the brain that are known to mediate the reproductive and non-reproductive effects of estradiol and that age influences the expression of ERβ mRNA in multiple, but not all brain regions. On the other hand, ERα mRNA levels appear to continue to be expressed at normal levels even in old animals in most brain regions and decreased only in the periventricular preoptic nucleus of old animals of all the specific areas of the hypothalamus that we examined. It is possible that the region-specific decline in receptor expression may be responsible for some of the decreased responsiveness to estradiol, but since this is not a global effect, other mechanisms are likely involved as well.

Acknowledgements

This work was partially supported by NIH AG02224 (P.M.W.) and AG05818 (M.E.W.).

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