ORIGINAL ARTICLE

Estrogen receptor-alpha immunoreactivity in the arcuate hypothalamus of young and middle-aged female mice

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Abstract

BACKGROUND: Changes in the neuroendocrine regulation of gonadal function, via altered hypothalamic sensitivity to peripheral hormones, are known to schedule reproductive maturation in the young and influence reproductive senescence. Estrogen (E) is a key hormone in this process. While changes in circulating levels of E over the life span are well documented, less is known about the corresponding changes in E sensitivity over the lifespan, especially during middle-age, when the initial signs of reproductive senescence emerge.

OBJECTIVE: Taking Estrogen Receptor (ER)-alpha-immunoreactive cells as an index of hypothalamic sensitivity to E, this investigation aims to quantify alterations occurring at middle age in comparison to young age.

METHODS: We counted ER-alpha-immunoreactive (IR) cells in the Arcuate hypothalamus of 6-week-old (young) and 18-month-old (middle-aged) C57BL/6J female mice, sacrificed at vaginal opening and diestrous, respectively. An automated imaging microscopy system (AIMS) was employed to generate counts of ER-alpha-IR cells for each sampled section of the Arcuate nucleus (ARC).

RESULTS: This study shows a 21% reduction in the number of ER-alpha-IR cells and an 18% reduction in total ARC cell populations with aging. However, the calculated percentage of ER-alpha IR cells is similar in both young and middle aged mice, 30% and 29%, respectively.

CONCLUSIONS: Both ER-alpha IR cell populations and total cell populations within the ARC hypothalamus decline by middle age in comparison to young age. Despite such a significant decrease in ER-alpha immunoreactive and total cells, both young and middle age mice maintain a similar ratio of ER-alpha IR cells to total cells in the ARC hypothalamus.

INTRODUCTION

The precise molecular mechanism and signaling transduction pathways that trigger puberty and changes seen in senescence have yet to be completely elucidated. What has been clearly established, is that altered hypothalamic feedback sensitivity is responsible for the shift in endocrine profiles that drive the target tissue changes associated with both puberty and aging. By triggering a cascade of shifting endocrine profiles, which in turn induce major alterations in the phenotype of target tissues, the hypothalamus, sitting atop the hierarchy of neuroendocrine influence, is ideally positioned to regulate both development and aging. Thus, in addition to maintaining homeostasis at any given point in the lifespan, the neuroendocrine system, comprised of the hypothalamus and anterior pituitary, drives the whole-organism phenotype through the lifespan.

Decades of aging research has yielded several theories of aging and methods of life extension suggesting neuroendocrine involvement in aging (Bartke *et al.* 2001; Hung *et al.* 2003; Wolkow *et al.* 2000; Yaghmaie *et al.* 2006). One variant of the neuroendocrine theories, referred to as the hypothalamic dysregulation hypothesis, postulates that changing hypothalamic sensitivity to endocrine feedback is the clock that times phenotypic change over the lifespan (Dilman *et al.* 1976). This theory proposes that aging is due to a "shift" in the sensitivity of the hypothalamus to specific hormones, with some hypothalamic nuclei becoming more, and others becoming less sensitive to circulating hormones. As a result of these changes in hormone sensitivity, the synchronized, pacemaker-like communication within the hypothalamic nuclei and between the hypothalamus and other neural and endocrine centers breaks down, disrupting the numerous endocrine, autonomic, & behavioral functions of the hypothalamus and resulting in the phenotypic changes associated with aging.

To the extent hypothalamic sensitivity is reflected in the number and distribution of cells expressing receptors for a particular ligand, this theory predicts a correlation between histological parameters and the expression of a youthful or aged phenotype. In other words, the number and distribution of feedback hormone receptors should change predictably over the lifespan.

This study has selected to count the hypothalamic receptors specific to estrogen (E) and evaluate them at very specific reproductive phenotypes: the stages of puberty and middle age. Changes in hypothalamic feedback sensitivity to E induce the endocrine shifts responsible for puberty (Ojeda *et al.* 2006) and reproductive senescence (Prior *et al.* 1998), two very distinct phenotypic events spanning both development and aging. Presuming estrogen receptors (ER) mediate hypothalamic sensitivity to E, and changes in E sensitivity are known determinants of puberty and reproductive senescence, changes in ER expressing cell populations should parallel and even precede these events.

Although E's actions are mediated by two ER subtypes, alpha and beta, this investigation is limited to measuring ER–alpha. While ER-alpha is expressed throughout the hypothalamus, the arcuate nucleus (ARC) is one of the most ER-alpha rich nuclei in the hypothalamus. More importantly, in vivo autoradiographic and immunocytochemical analyses have shown that only scattered regions of the ARC express ER-beta while ER-alpha-expressing cells heavily populate this region (Merchanthaler *et al.* 2004). Similarly, knockout studies show a marked decline in ARC E sensitivity when ER-alpha genes expression is blocked (Shughrue *et al.* 1998). Thus, in the ARC, from both a localization and functional perspective, tracking the ER-alpha subtype, rather than the beta-subtype, seems more appropriate.

The functional role of ARC ER-alpha cells in the regulation of reproductive function remains under investigation but much has been elucidated. Early studies using estrogen exposure showed that the ARC was the hypothalamic nucleus most sensitive to estradiol in the inhibition of ovulation (Lisk *et al.* 1973). Similarly, early ARC ablation studies showed that the ARC was essential for the maintenance of basal levels of gonadotropic hormone (Carpenter 1983). More recently, investigations of hormone induced synaptic plasticity in the ARC have shown a robust reversible decline in ARC synapses occurring during the gonadotropin surge (Naftolin *et al.* 2007), suggesting a possible morphological basis for the ARC's role in the cyclical regulation of reproductive function.

Interestingly, studies have shown that the ARC, though void of gonadotropin releasing hormone (GNRH) expression, modulates the gonadotropin surge indirectly by suppressing pro-opiomelanocortin (POMC) expression in axons projecting to GNRH neurons of the pre-optic area (POA), (Leranth *et al.* 1988). Thus, the ARC, though void of GNRH, conveys estrogen sensitivity to the POA by suppressing POMC expression in neurons, which in turn modulates the gonadotropin surge. Furthermore, with aging, this estradiol induced suppression of POMC in the ARC is abolished, (Petersen *et al.* 1993; Weiland *et al.* 1992). This suggests that a functional consequence of dwindling ARC ER-alpha cells may be to diminish ARC influence on the timing of the gonadotropin surge, thereby altering reproductive function with age.

The ARC is also thought to play a significant role in development. Recent work has identified a novel signaling pathway involving the G protein-coupled receptor-54 and its natural ligand, Kisspeptins, which research suggests serves as the molecular mechanism that reawakens GnRH neurons at puberty (Morelli *et al.* 2008). Kisspeptins have been shown to be negatively regulated by the administration of estradiol to the ER-alpha rich arcuate nucleus (Li *et al.* 2007). Thus providing an additional molecular mechanism for the role of hypothalamic feedback sensitivity to in regulating the onset of puberty.

Given the functional implications of the ARC in both reproductive development and aging, this investigation compares the ER-alpha-IR sub-populations and the total cell population of the ARC at two distinct points in the female C57BL/6J mouse lifespan: (1) The onset of puberty (6 weeks), as measured by vaginal opening and (2) At diestrus in cycling late-middle age (18 months), as measured by vaginal smears. These two age groups represent very specific and easily identifiable phenotypic events that differ with respect to reproductive function. Because reproductive function is largely determined by the endocrine profile and the endocrine profile is largely determined by the hypothalamus, differences in reproductive function should, in turn, be reflected in the ER alpha expressing cells with in the ARC hypothalamus.

MATERIALS AND METHODS

Animals

Animal care and all procedures involving live animals were conducted in accordance with the standards set fourth by, and with the approval of, the UC Berkeley Animal Care and Use Committee. All mice used in this study were purchased from Jackson Laboratories (Bar Harbor ME, USA) and housed 4 to a cage in barrier facilities, (lights on 0700 h, off 1900 h). Seven virgin C57BL/6J mice were obtained at 3 weeks of age and sacrificed at 6 weeks at vaginal opening. Seven C57BL/6J retired breeders were obtained at 10 months and sacrificed at 18 months. The cyclicity of middle-aged mice was monitored via vaginal smears and they were sacrificed during diestrus. The young mice were sacrificed at 6 weeks of age upon the onset of vaginal opening marked by daily inspection.

Tissue Preparation

All mice used in this study were sacrificed following an adequate adaptation period in barrier facilities. Mice were injected with the anticoagulant Heparin, followed by the anesthetic KAX (ketamine, acepromazine, and xylazine). Aortic cannulation was performed and the mice were perfused with 10 mL of Hextend®, a physiologically balanced plasma volume expander, followed by 20 mL of neutral buffered formalin (NBF). This ensured optimal blood washout and fixation. The brains were then removed and stored in NBF for 12 hours prior to gradual dehydration in serial immersions of progressively stronger solutions of cellosolve, an organic solvent that serves as a medium for wax infiltration. A 4 mm coronal slice encompassing the hypothalamus was then excised and infiltrated with polyester wax (MP=37 °C), followed by further infiltration and embedding in 80:20/polyester: ester wax (MP=42 °C).

Comparative serial sectioning

Following wax embedding, each coronal slice containing the hypothalamus was serially sectioned on a Leica Microtome. Each specimen was microscopically aligned and fixed in the same reference position prior to serial cross sectioning to acquire anatomically matched hypothalamic tissue from all brains. This microscopic alignment was done by fixating at specific landmarks appearing on the boundaries of all three geometric planes. Detailed methodology on reaching this microscopic alignment has already been published (Yaghmaie *et al.* 2005). After proper alignment was achieved, specimens were locked in place, and serial cross-sections were collected and immuno histochemically stained.

Fig. 1. A - Photomicrographs of ER-alpha immunoreactive and ER-alpha non immunoreactive cells in the Arcuate hypothalamus of female mice. Photomicrograph of a high powered micrograph (x600) showing ER-alpha immunoreactive (solid arrow) and ER-alpha non immunoreactive cells (dashed arrow). ER-alpha non immunoreactive cells are counterstained with ethyl green. Such cross sections were subjected to immunohistochemical staining using the ABC (avidin biotin-peroxidase complex) method. Subsequent to citrate-buffered antigen retrieval, sections were incubated in 1.5% goat serum in phosphate-buffered saline to prevent non-specific binding, followed by incubation in a 1:1 200 dilution of a rigorously validated rabbit anti-ER-alpha polyclonal antibody in 1.5% goat serum. Each cross sections was then incubated in a biotinylated anti-rabbit secondary antibody. For further amplification of the immunohistochemical stain, the sections were incubated in a streptavidin horseradish peroxidase and then visualized through treatment with a chromogen peroxidase substrate kit.
B - Photomicrograph of ER-alpha non immunoreactive cells (x600) identified by an ethyl green counter stain. No ER-alpha immunoreactive cells are identified with out the presence of the rabbit anti-ER-alpha polyclonal antibody.

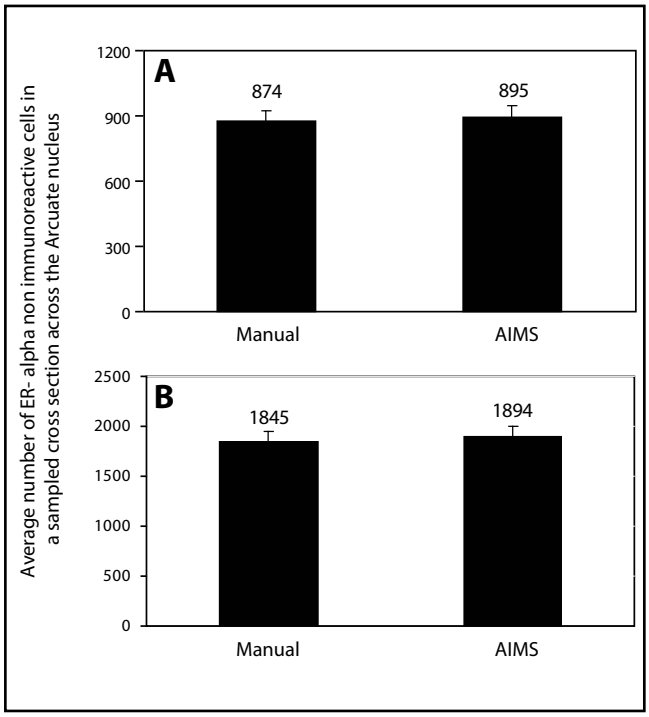

Fig. 2. To ensure the validity and reliability of AIMS, 25% of the cross-sections for experimental groups were manually counted at 600× magnification by eye and the average value of each category of cells per sampled cross-section was compared. As shown in Figure 2, manual counts of both ER-alpha-immunoreactive and ER-alpha-non-immunoreactive cells from sampled cross-sections correspond very closely to values generated by AIMS, thereby ensuring reliability of all cell counts generated by AIMS. Cell counts generated by manual vs. AIMS. Counts are average values from samples cross section across the Arcuate nucleus. Panel A shows no significant difference in detecting ER-alpha immunoreactive cells manually in comparison to AIMS. Panel B shows that manual cell count of ER-alpha non immunoreactive cell are similar to values acquired by AIMS.

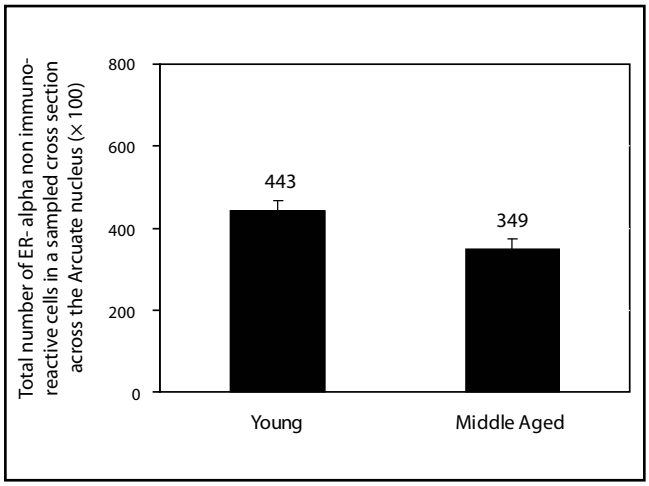

Fig. 3. Total number of ER-alpha immunoreactive cell counts, with standard error bars, across the sampled Arcuate nucleus of Young and Middle Aged mice. Middle Aged mice show a statistically significant 21% decline in ER-alpha immunoreactive cells in comparison to young mice (*p<0.05). Data are based on cross sections collected at 12 micro meter intervals across the Arcuate nucleus of seven mice in each group.

Immunohistochemical Staining

Anatomically aligning brain tissue as described above made it possible to cross section each mouse in the same anatomical region to obtain comparative samples. To localize and immunostain the ARC nucleus, the caudal boundary was marked at the fusion of the initial Infundibular stalk with the base of the brain. Starting from this caudal boundary, 30 cross sections, each 4 micrometers thick, were immunostained in the rostral direction, at 12 micrometer intervals. Thus, a total of approximately 480 microns of ARC nucleus was surveyed per brain. This region spanned the caudal-rostral axis from the initial fusion of the Infundibular stalk with the base of the brain to the terminus of the ventromedial nucleus. A mouse brain atlas was utilized to assist in establishing neuro anatomical markers (Paxinos *et al.* 2001). Cross sections were immunostained for nuclear ER-alpha. The sections were stained using the ABC (avidin biotin-peroxidase complex) method. This method is further described in the legend of Figure 1.

Data Analysis

A novel automated imaging microscopy system (AIMS) was developed for use in this study. The system is comprised of 4 main components: the computer hardware, the AIMS software, the electronic interface that allows the computer to manipulate the microscope stage and fine focus, and the automated digital image capturing microscope.

Cell Counts

The Automated Imaging Microscopy System (AIMS) was employed to generate counts of ER-alpha- immunoreactive (IR) cells and the total cell population for each sampled section of the Arcuate. From every coronal section, AIMS digitally stored 64 fields of view captured at 600× magnification, covering the entire ARC. The fields were then assembled into single images of the ARC in that section plane and analyzed by a cell counting program. The counting program identified cells as either immuno-positive or negative in each composite image and generated cell counts for each section (Figure 1). In addition to eliminating the need for selective sampling, automated counting compares favorably with other methods of histological quantification in terms of the ratio of time expended to data obtained. To ensure the validity and reliability of AIMS, manual counts by eye and AIMS were compared and both groups maintained similar cell counts as shown in Figure 2. The validity AIMS was established in previous investigations (Saeed *et al.* 2007; Yaghmaie *et al.* 2006). Cell counts were generated from each mouse brain (7 young and 7 middle-aged), and provided two values for each serial section analyzed: 1) the number of ER alpha-IR cells and 2) the Total number of ARC cells, derived by adding together ER-alpha immunoreactive and ER-alpha non immunoreactive cells

Statistical analyses

Statistically significant differences in counts of ER-alpha immunoreactive cells and all stained cells in the ARC between Young vs. Middle Aged mice were found by using t-tests. All statistical calculations are done by Graph Pad In Stat 3.0 software. Differences are considered statistically significant at $p<0.05$.

RESULTS

Cell counts generated from cross sections, taken at regular 12-micrometer intervals across the ARC nucleus of young and middle-aged mice show a decline in the number of ER-alpha immunoreactive cells with aging. Middle-aged mice show a statistically significant 21% decline in ER-alpha immunoreactive cells when compared to their young counterparts (Figure 3). Aging also shows a significant loss of total cells within the ARC nucleus as middle-aged mice exhibit an 18% decline in total number of cell in comparison to young mice (Figure 4). Given these trends of cellular decline with age, we also calculated the percentage of ER-alpha immunoreactive cell in the Arcuate nucleus. The calculated percentage of ER-alpha is similar in both young and middle aged mice, 30% and 29%, respectively (Figure 5).

In summation, three general conclusions can be made about the Arcuate nucleus of young (6 weeks) and middle-aged (18 months) female C57BL/6J mice:

1) ER-alpha-immunoreactive (IR) cell-populations significantly decreased by middle age.
2) Total ARC cell populations significantly decrease by middle age.
3) Despite a significant decrease in ER-alpha immunoreactive and total cells, both young and middle age mice maintain a similar calculated percentage of ER-alpha immunoreactive cells in the ARC hypothalamus.

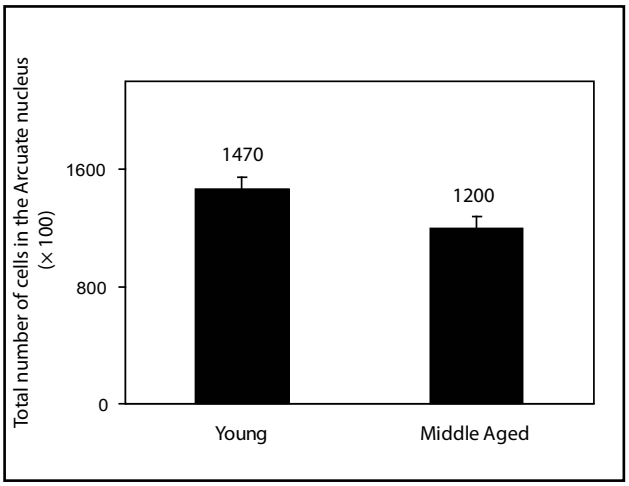

Fig. 4. Total number of cells, with standard error bars, across the sampled Arcuate nucleus of Young and Middle Aged mice. Middle Aged mice show a statistically significant 18% decline in total cells in comparison to young mice (*$p<0.05$). Data are based on cross sections collected at 12 micro meter intervals across the Arcuate nucleus of seven mice in each group.

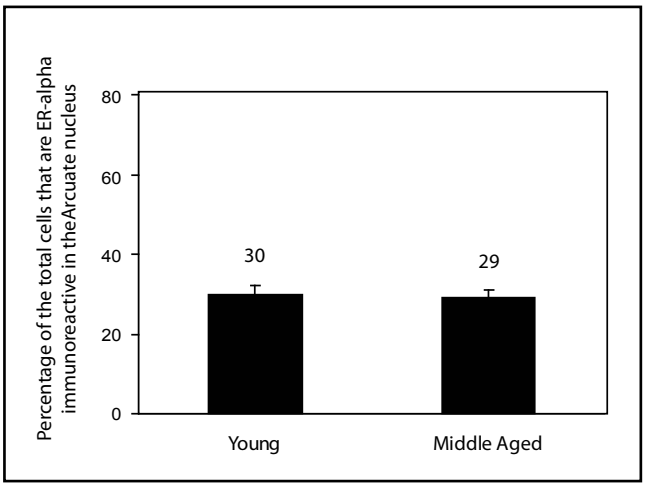

Fig. 5. Calculated percentage of total cell that the ER-alpha-immunoreactive, with standard error bars, across the sampled Arcuate nucleus of Young and Middle Aged mice. Middle Aged mice maintain a similar percentage of ER-alpha immunoreactive cells in comparison to young mice. Data are based on cross sections collected at 12 micro meter intervals across the Arcuate nucleus of seven mice in each group.

DISCUSSION

This investigation shows a significant age-related decline in the number of estrogen receptor-alpha immunoreactive cells and the total cell population within the arcuate (ARC) hypothalamus. These findings are consistent those of others using similar immunohistochemical techniques, as well as autoradiography and *in vitro* binding studies (Chakraborty *et al.* 2004). However, because our study employed the use of an automated image microscopy system (AIMS), we were able to survey the ARC in a much more thorough manner, yielding a more accurate analysis of ARC cell numbers. While decreases in Total ARC cells have been reported previously (Hsu *et al.* 1978), our fine sampling (every 12 microns) provided improved quantification of the ER-alpha-IR cell numbers in the ARC.

The functional significance of an apparently stable ratio of ER expressing cells to the rest of the ARC cell population is not clear, but may serve to maintain the role of E-sensitivity in the ARC. Thus, maintaining E-sensitive cell populations in the ARC may be crucial.

Because of the tremendous redundancy and connectivity of neuronal tissue, the functional impact of localized cell loss is often insignificant or undetectable until major cell loss is apparent. However, our findings that ER-alpha-IR cells in the ARC decline with age may be the mechanism by which corresponding estrogen-mediated actions localized to the ARC change with age. Moreover, in a previous investigation our group found a similar decline in ER-alpha-IR cells in the Pre-Optic

hypothalamus, which is intimately involved in regulating central reproductive senescence (Yaghmaie *et al.* 2005). Thus the age related loss of ER-alpha-IR cells is not specific to the ARC and could reflect a global decrease in hypothalamic estrogen sensitivity.

Estrogen becomes progressively less able to elicit responses from aged tissues (Weiss *et al.* 2004), and ER mediates most of the effects of E, suggesting decreases in cells expressing ER may be the mechanism that underlies reduced sensitivity to E. This may be true in the hypothalamus, where responsiveness to the neuroprotective actions of estradiol decreases dramatically by the time animals reach middle-age (Wise *et al.* 2001) and specifically in the ARC where age abolishes the estradiol-induced suppression and diurnal rhythm of proopiomelanocortin (POMC) expression (Weiland *et al.* 1992). In both cases, age related declines in ARC ER-alpha-IR cell numbers may lead to reduced E sensitivity, thus resulting in the reduction of these estradiol mediated actions.

Given the phenotypic backdrop of puberty and middle-age, the functional impact of changing patterns of ER expression in the ARC can be explained within the framework of what is currently known about the ARC's role in reproductive function. Decreased ARC estrogen sensitivity with age shown here could abolish the estradiol-induced suppression of POMC expression in ARC neurons projecting to GNRH neurons of the preoptic nucleus. Through such a mechanism the luteinizing hormone surge, necessary for ovulation, could be potentially modified, causing the changes in the estrous cycle associated with reproductive senescence. Although the middle-aged mice in this study were not yet exhibiting altered cycles typical of reproductive senescence, one would expect changes in hypothalamic estrogen sensitivity to precede altered reproductive function.

Furthermore, the functional impact of decreased ER-alpha-IR cells can be discussed in light of the hypothalamic dysregulation hypothesis of aging. As noted earlier, this theory suggests that altered hypothalamic sensitivity to endocrine feedback is the clock that times phenotypic change over the lifespan. If this is true, altering ARC E-sensitivity with age may be a contributing factor. Because of the ARCs central location in the hypothalamus and its proximity to the hypophysial portal system, decreased ARC E-sensitivity may modulate neurosecretions from other hypothalamic nuclei into the portal system, thereby altering the endocrine profile, and contributing to endocrine driven phenotypic changes associated with aging.

Despite these possible functional roles for E sensitivity in the ARC, several limitations to the current study are evident. For example, we cannot determine from the present study whether ER-alpha cells are actually lost in the older age group. The observed decline in ER-alpha-IR cells may be due to age related changes in the receptor itself. Altered ER epitopes may result in decreased immunoreactivity, and thus a decline in ER-alpha-IR cells.

Furthermore, since our stain was for nuclear ER-alpha, it may be possible that the decline in ER-alpha-IR cells may simply reflect altered ER translocation dynamics rather than actual cell loss. Age related declines in E-induced nuclear ER concentrations have been demonstrated in middle-aged rats (Belisle *et al.* 1983), thus there may not be a decline in ER numbers, but rather less ER being translocated to the nucleus. Similarly, rather than the actual loss of ER-alpha expressing cells, an age-related down-regulation of ER-alpha expression may be occurring. This would decrease the number of cells expressing ER-alpha and therefore decrease the number of ER-alpha-IR cells detected in the ARCs of older mice.

In addition, by using the expression of Estrogen receptor–alpha as the sole index of E-sensitivity, our findings are blind to the potential effects of secondary messengers and transcription factors in the E signaling pathway. Measurement of a decreased downstream functional product within the E signaling pathway would further affirm and connect our findings of decreased ER-alpha IR cells to a decline in E sensitivity and signaling in the ARC nucleus.

However, despite these limitations and assuming the loss of ARC ER-alpha cells does occur, a possible mechanism for their loss may be E toxicity. An ovary-dependent, age related degeneration in the ARC nucleus (Hung *et al.* 2003), suggests that cyclic surges of endogenous estradiol may lead to the gradual production of ARC lesions. Since ER containing cells in the ARC would be most sensitive to E, their loss would be expected.

In conclusion, this study shows that in as early as 18 months, declines occur in the ER-alpha-IR cells and in the total cell population of the ARC hypothalamus of female mice and that younger, 6 week old mice, have an average of 21% more ER-alpha-IR cells and 18% more cells in the total ARC cell population, than in their older counterparts. Thus, E-sensitivity, as measured by ER-alpha immunoreactivity, declines with age, and this decrease precedes reproductive senescence. The molecular basis for this decline and the role of decreased ARC sensitivity in altered responsiveness to E and reproductive function remains to be determined. However, descriptive biological studies such as this investigation can profile neuroendocrine biomarkers that change with aging and such a road map can pave way for clinical interventions through stem cells and genetic manipulation to regenerate cell populations present in young age.

In addition, traditional avenues of preventative and therapeutic hormone replacement continue to evolve and characterizing tissue specific sensitivity to hormones is a necessary step towards understanding the relationship between circulating levels of hormones and desired phenotypic outcomes. With time, such an understanding could result in more effective and more precisely targeted interventive medical procedures

designed to delay the senescent phenotype by manipulating hormone profiles and tissue specific hormonal receptor expression.

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DISCLOSURE STATEMENT

The authors of this manuscript have no financial conflicts or interests to disclose.

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