Caloric restriction reduces cell loss and maintains estrogen receptor-alpha immunoreactivity in the pre-optic hypothalamus of female B6D2F1 mice

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ORIGINAL ARTICLE

Abstract

Life-long calorie restriction (CR) remains the most robust and reliable means of extending life span in mammals. Among the several theories to explain CR actions, one variant of the neuroendocrine theories of aging postulates that changing hypothalamic sensitivity to endocrine feedback is the clock that times phenotypic change over the life span. If the feedback sensitivity hypothesis is correct, CR animals should display a significantly different pattern of hormone-sensitive cell density and distribution in the hypothalamus. Of the many endocrine signal receptors that may be involved in maintaining hypothalamic feedback sensitivity, our study has selected to begin mapping those for estrogen (E). Altered hypothalamic sensitivity to E is known to schedule reproductive maturation and influence reproductive senescence. Taking estrogen receptor-alpha (ERα) immunoreactivity as an index of sensitivity to E, we counted ERα immunoreactive and non-immunoreactive cells in the pre-optic hypothalamus of young (6 weeks), ad-libitum (Old-AL) fed old (22 months), and calorie restricted (Old-CR) old (22 months) female B6D2F1 mice. An automated imaging microscopy system (AIMS) was used to generate cell counts for each sampled section of pre-optic hypothalamus. Results show a 38% reduction in ERα immunoreactive cells and an 18% reduction in total cell numbers in AL-old mice in comparison to young mice. However, CR mice only show a 19% reduction in ERα immunoreactive cells and a 13% reduction in total cell numbers in comparison to young mice. This indicates CR prevents age-related cell loss and maintains estrogen sensitivity in the pre-optic hypothalamus of old female B6D2F1 mice.

Abbreviations and Units

ABC: Avidin Biotin-Peroxidase Complex
AIMS: Automated Imaging Microscopy System
AL: Ad-Libitum
CR: Caloric Restriction
E: Estrogen
ERα: Estrogen Receptor-alpha
ERβ: Estrogen Receptor-beta
KAX: Ketamine, Acepromazine, Xylazine
LH: Lutenizing Hormone
mL: milliliter
mm: millimeter
MP: Melting Point
MPOA: Medial Pre-Optic Area
NBF: Neutral Buffered Formalin
NIA: National Institute on Aging
SERMs: Selective Estrogen Receptor Modulators

Introduction

Life-long caloric restriction (CR) extends rodent life span by as much as 50% [1–4], but 70 years after its physiological characterization, CR's underlying mechanism remains unresolved. During this time, alternate methods of life span extension using endocrine manipulation have been successfully implemented [5, 6] and research perspectives suggesting that the hypothalamus plays a role in aging have been growing [7]. This has led many to speculate CR's protective affect is mediated by neuroendocrine mechanisms [8–10], however, experimental designs yielding information on the causal relationship between neuroendocrine biomarkers and the anti-aging action of CR have been lacking.

Therefore, we have begun a series of investigations that aim to map endocrine receptor profiles of the hypothalamus across the life span under conditions of ad-libitum feeding and CR. By mapping these receptor profiles we hope to identify the key neuroendocrine responses to CR so that further interventional studies can be designed to test the extent to which these neuroendocrine responses to CR mediate the effects of CR on life span [11].

Of the long list of steroid and peptide hormone receptors expressed in the hypothalamus, three have been targeted: Estrogen Receptor-alpha (ERα), Growth Hormone Receptor, and the Insulin-Like Growth Factor-1 Receptor. These receptors were chosen because they mediate hypothalamic feedback sensitivity to endocrine signals involved in regulating three distinct, but interdependent, physiological processes: reproduction (estrogen), growth and anabolic maintenance (growth hormone) and energy metabolism (insulin). In addition, each hormone for these receptors shows a distinct profile of change in pattern of secretion, basal level, or total tissue exposure over the life span, and each shows a distinct attenuation or reversal of direction in this profile under the influence of CR [12].

In this investigation we present an analysis of ERα immunoreactivity in the pre-optic hypothalamus of young (6 week) & old (22 month) ad-libitum fed and old (22 month) CR mice. More specifically, we counted cells in the medial pre-optic area (MPOA), which includes the medial pre-optic nuclei, central pre-optic nuclei, and surrounding ependymal cells of the third ventricle. ERα immunoreactivity is taken as an index of estrogen (E) sensitivity. Altered hypothalamic sensitivity to E is known to schedule reproductive maturation and influence reproductive senescence [2, 3], two phenotypic events delayed by CR [13, 14].

Two ER subtypes, alpha and beta, mediate E's actions. This investigation is limited to measuring ERα because of its distribution and associated functions. In contrast to ERβ (β), which is broadly expressed throughout the central nervous system, ERα's strongest expression is limited to the amygdala and hypothalamus [15], our area of interest. In addition, ER knockout studies have shown that ERα, not β, plays a critical role in the maintenance and regulation of reproductive function [16] and in estradiol mediated protection against brain injury [17]; suggesting ERα is the target of Selective Estrogen Receptor Modulators (SERMs), now used in E-replacement therapies to treat and prevent CNS injuries [18].

Although ERα is expressed throughout the hypothalamus, this investigation is limited to the medial pre-optic area (MPOA) of the hypothalamus. The MPOA is one of the most ERα rich cell populations in the hypothalamus and has been identified as the "pulse generator" necessary for the cyclic surge of lutenizing hormone (LH) that precedes ovulation. In addition to this critical role in regulating reproductive function, the MPOA also plays a key role in the regulation of blood pressure & body temperature, two physiological parameters that decrease with CR [13].

Materials and Methods

Animals

Young, Old-Caloric-restricted (Old-CR) and Old-Ad-libitum fed (Old-AL) B6D2F1 female mice were purchased from the National Institute on Aging (NIA), and individually housed in barrier facilities (lights on 0700h, off 1900h), in accordance with the standards set fourth by the Berkeley Animal Care and Use Committee. Young-AL mice were sacrificed at 6 weeks upon vaginal opening. CR was initiated at 14 weeks of age at 10% restriction, increased to 25% restriction at 15 weeks, and finally to 40% restriction at 16 weeks where it was maintained for the duration of life. Both CR and Old-AL mice were sacrificed at 22 months during diestrous as determined by vaginal smears.

Tissue Preparation

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 10mL of Hextend, a physiologically balanced plasma volume expander, followed by 20mL of neutral buffered formalin (NBF). This ensured optimal washout and cell death by 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 4mm 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). These methods and the use of low-melting point infiltration media were exploited to maximize preservation of tissue integrity and minimize loss of antigenicity.

Comparative serial sectioning

Subsequent to being embedded in wax each mouse brain was serial sectioned on a Leica microtome. To ensure 3 dimensional spatial equivalence for the upcoming stereological analyses, every mouse brain was microscopically adjusted and fixed in the same reference position prior to being cross sectioned. This alignment procedure was done by using specific anatomical landmarks that appeared at the boundaries of all three planes that correspond to the reference position. More precisely, the microtome allowed us to selectively rotate the brain in either x–y, y–z, or x–z planes to locate particular landmarks in all three 2 dimensional planes, leading to an indirect but reproducible 3 dimensional adjustment of the brain before sectioning (on a given cross section in this study, x axis = medial/lateral motion, y axis = rostral/caudal motion, and z axis = dorsal/ventral motion). Although the region of interest in this study, namely the medial pre-optic area (MPOA), lies in the rostral portion of the hypothalamus, serial sectioning was done in a caudal to rostral direction, so the reference position located before sectioning actually matches a relatively caudal hypothalamic location shown on figure 51 in the mouse atlas of Paxinos [19]. The x–y planer adjustment was done by lining up the left and right ventral tips of Ammon's horn-field CA3, as is shown in figure 51 of Paxinos' mouse atlas. The y–z planar adjustment was done on the dorsal end by locating the beginning of corpus collosusm crossing and on the ventral end by reaching the caudal opening of the mammillary recess. And lastly, the x–z planar adjustment was done by reaching a strict symmetrical appearance on both sides of the z axis. Subsequent to proper alignment, the brain was locked in position and serial cross sections of 4 micrometer thickness were collected in a caudal to rostral direction for the entire length of the hypothalamus. Then each 4 micrometer cross section was permanently mounted on a glass slide.

Immunohistochemical Staining

As a result of the 3 dimensional brain positioning protocol described above, all mice brains were cross sectioned in the same anatomical position. To immunostain the pre-optic hypothalamus, we picked the rostral boundary for each mouse brain at the very beginning of the crossing of the anterior commissure and then further immunostained cross sections at every 32 micrometers in the caudal direction, until a total of seven anatomically and spatially equivalent sections were stained for each mouse. More specifically, the rostral-caudal boundaries of the immunostained region in this study are 4.000–3.776 mm anterior to the interaural line in reference to the mouse brain atlas of Paxinos [19]. This region expands a total of 224 micrometers caudal from where the anterior commissure crosses first. Figure 1 shows two of the seven levels that were sampled in the pre-optic hypothalamus, along with corresponding views from a mouse atlas [19]. In all, a theoretical total of 35 spatially equivalent cross sections should be obtained from each group of mice (5 animals in each group of Young, Old-AL, and Old-CR mice multiplied by seven immunostained cross sections per animal). However, due to tissue damage only 31 cross sections were immunostained from each group.

Immunostaining for nuclear ERα was done by 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 1:1200 dilution of rabbit anti-ERα polyclonal antibody (200 micrograms/1 mL of PBS; Santa Cruz BioTechnology, Inc., Santa Cruz, CA) in 1.5% goat serum. The validity of this antibody is confirmed through its successful usage in numerous studies [20, 21]. The sections were then incubated in a biotinylated anti-rabbit secondary antibody. For further amplification sections were incubated in a streptavidin horseradish peroxidase and then visualized through treatment with a chromogen peroxidase substrate and an ethyl green counterstain. Immunostaining was conducted in batches consisting of anatomically equivalent sections from all three groups of mice, thus removing staining conditions as a variable in cell count comparisons.

Data Analysis

Each immunostained cross section is scanned in an Automated Imaging Microscopy Analysis System (AIMS) [22], using a x60 objective at the fixed illumination. For each section, AIMS digitally stores 384 fields of view captured at 600X magnification, and assembles fields captured from each section into a single image of the pre-optic region in that section. Stereological analysis happens through outlining the medial pre-optic area (MPOA), which contains the medial pre-optic nuclei, central pre-optic nuclei and the third ventricle with surrounding ependymal cells (MPOA is outlined in Figure 1). To allow stereological comparisons AIMS places this outline enclosing the MPOA in each of the seven cross sections examined. In essence, AIMS constructs an outlined region of space with dimensions of 1167μm × 224 μm x 1000 μm (*x* x *y* x *z*, these dimensions are only approximations of the actual non-geometric 3 dimensional MPOA region), by stacking 7 evenly spaced sections at 32 micrometers intervals for every mouse brain. This 3 dimensional region contains the rostral 224 micrometers the medial pre-optic area

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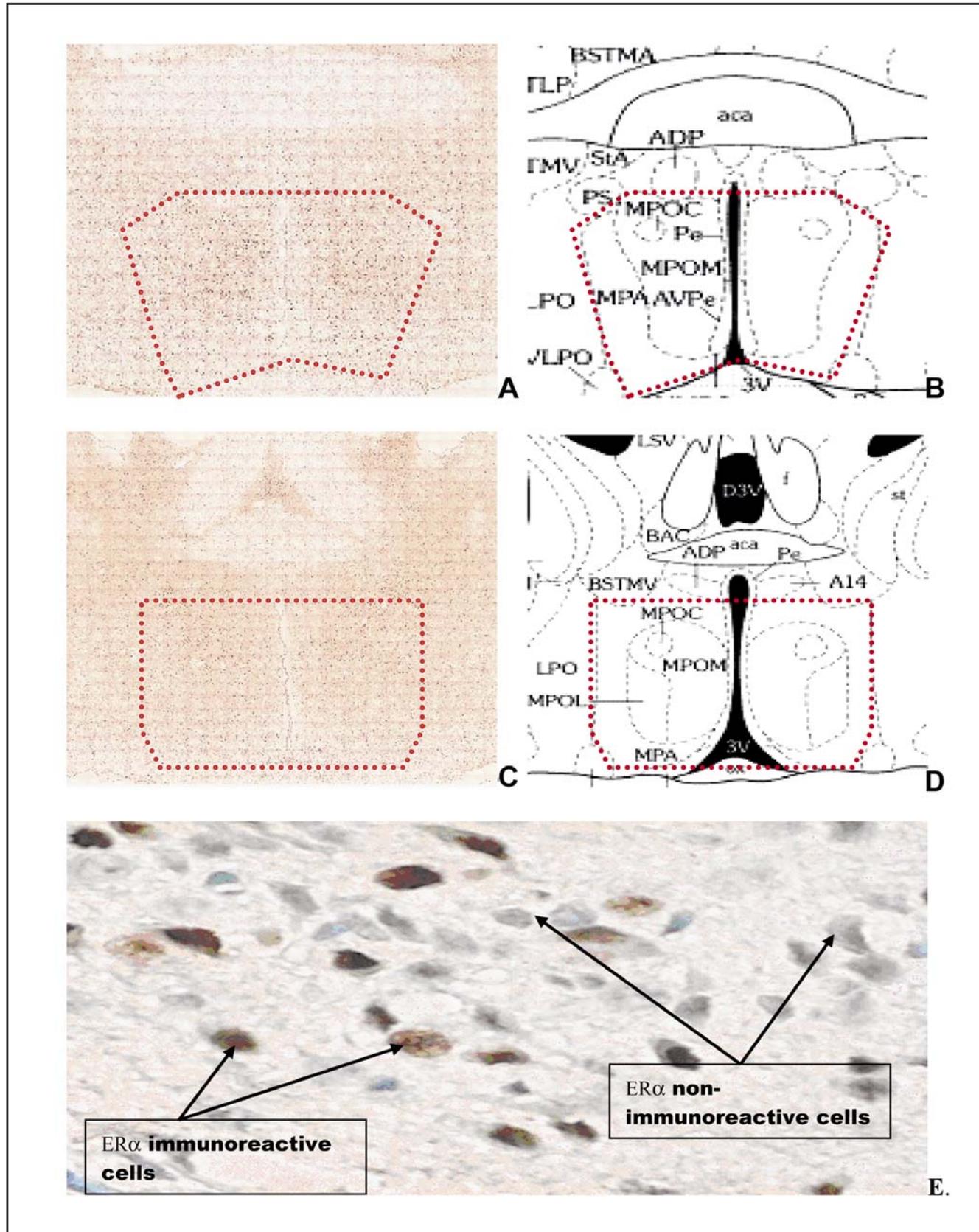
Figure 1: Photomicrographs of ERα immunoreactive and ERα non-immunoreactive cells in the MPOA of female mice. A and C are photomicrographs of mice brain tissue showing the MPOA, and its inclusive nuclei, outlined in red at anatomical levels of 96 micrometers and 224 micrometers caudal to where the anterior commissure crosses first, respectively. B and D are modified images from a mouse brain atlas that correspond to levels A and C, respectively. Although A and C show MPOA tissue at only two levels caudal to the initial crossing of the anterior commissure, the analyses in this study are based on counting cells at seven levels of the MPOA. Each counted level of MPOA is 32 micrometers from the other, spanning a total of 224 micrometers caudal from where the anterior commissure crosses first. E is a high power micrograph (600x) showing ERα immunoreactive and ERα non-immunoreactive cells that were appropriately classified and counted by AIMS. Non-immunoreactive reactive cells are counterstained with ethyl green.
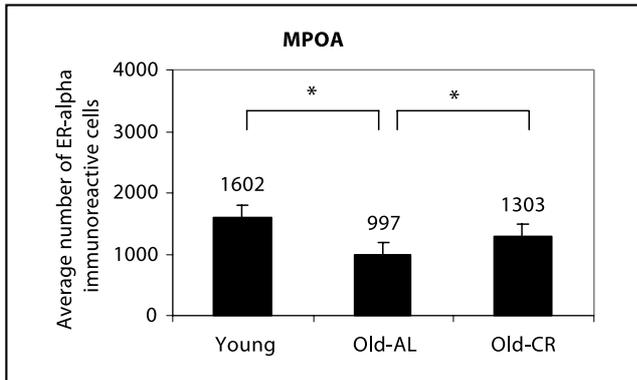
Figure 2: Averages of ERα immunoreactive cell counts, with standard error bars, in the rostral 224 micrometers of MPOA from Young, Old-AL and Old-CR. Old-AL mice show a 38% decline in ERα immunoreactive in comparison to young mice (* implies p<0.05). Old-CR mice show a 31% increase in the average number of ERα immunoreactive cells in comparison to Old-AL mice (p<0.05). Data are based on 3l cell counts across the MPOA of 5 mice in each group.
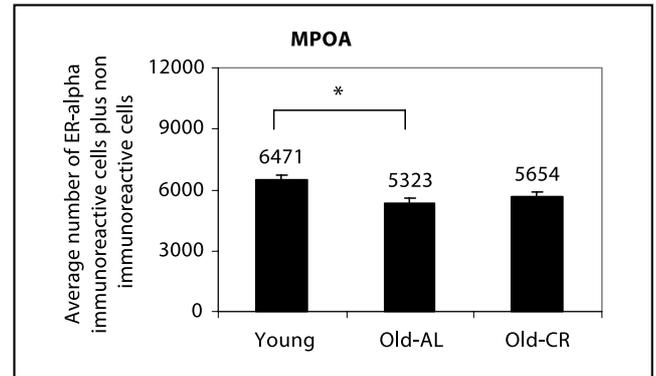
Figure 3: Averages of ERα immunoreactive plus non-immunoreactive cell counts, with standard error bars, in the rostral 224 micrometers of MPOA from Young, Old-AL and Old-CR mice. Old-AL mice show an 18% cell loss compared to Young mice (* implies p<0.05). Old-CR mice show a 6% increase in the average number of ERα immunoreactive plus non-immunoreactive cells in comparison to Old-AL mice, but these data are not statistically significant (p>0.05). Data are based on 3l cell counts across the MPOA of 5 mice in each group.

(MPOA). An unbiased counting program then identifies and classifies cells in the outlined MPOA region in each cross section, through analyzing and comparing pre-set color intensity thresholds, as immuno-positive or negative in each composite image. Figure 1 shows immuno positive (dark brown color) and negative cells (blue green color), which are appropriately counted by AIMS. From a total of 31 cell counts in each group (up to seven counts per animal and five animals in each group), average values of ERα immunoreactive cells and non-immunoreactive plus immunoreactive or all stained cells in the MPOA are calculated and compared below. Further information on AIMS can be viewed at www.arclab.org/AIMS/.

Statistical Analysis

Differences in the average values of ERα immunoreactive cells and average values of ERα immunoreactive cells plus non-immunoreactive cells between Young, Old-AL and Old-CR mice in the MPOA are determined by using t-tests. More precisely, t-tests are done separately for Young vs. Old-AL mice to isolate effects of aging, and for Old-CR vs. Old-AL mice to isolate the effects of caloric restriction. These statistical calculations are done by the GraphPad InStat 3.0 software. Differences are considered significant at p<0.05.

Results

An analysis of the average cell counts in the rostral 224 micrometers of the MPOA shows a decline in ERα immunoreactive cell populations with age, with Old-AL mice exhibiting a 38% drop in ERα immunoreactive cells compared to young mice, *p*<0.05 (Figure 2). In addition, averages of ERα immunoreactive plus non-immunoreactive cell counts in Old-AL mice demonstrate an 18% cell loss compared to young mice, *p*<0.05 (Figure 3). These results indicate

that the rostral MPOA undergoes extensive cell loss with aging, especially in the ERα immunoreactive cell populations.

The age-related cell loss in the ERα immunoreactive cells of the MPOA is moderately attenuated with CR. On average, Old-CR mice exhibit 31% more ERα immunoreactive cells than their age-matched Old-AL counterparts, p<0.05 (Figure 2). In addition, averages of ERα immunoreactive plus non-immunoreactive cell counts increase from Old-AL mice to Old-CR mice, by 6%, but this difference is statistically insignificant, p>0.05 (Figure 3). These results indicate that caloric restriction increases cell numbers, especially in ERα immunoreactive cell populations of the MPOA.

Discussion

This investigation's report that estrogen sensitivity and cell numbers in the pre-optic hypothalamus decline with age, confirms the findings of other studies using similar immunohistochemical techniques, auto-radiography and in-vitro binding [23–25]. However, by using an automated imaging microscopy system (AIMS), this analysis provides a more accurate measurement of these changes and maps their relative distribution through the medial pre-optic area (MPOA).

More importantly, this study includes an analysis of changes in the pre-optic hypothalamus of age-matched caloric restricted mice. This analysis demonstrates that age-related changes in the MPOA are attenuated or suppressed by caloric restriction, an observation which has, to our knowledge, not been reported elsewhere. Furthermore, by tracking the change in both estrogen receptor (ER) expressing cells and the general cell population (ERα immunostained plus non-immunostained cells) simultaneously, this analysis provides the ratio of the estrogen sensi-

tive sub-population to the general cell population of the MPOA, and reveals that the maintenance of this ratio is especially preserved by caloric restriction.

We cannot determine from this present investigation whether the measured decline in ERα expressing cells represents cell death or a coordinated down-regulation of estrogen sensitivity with age. Furthermore, because our immunostain is specific to nuclear bound ERα, altered ERα translocation dynamics, known to occur with aging [26], or simply altered ERα antigenicity, may have exaggerated the apparent loss of ER expression. Similarly, because our stain was not specific to neurons, but rather brain cells in general, the actual magnitude of neuronal cell loss may be obscured by glial cell numbers, which increase with age [27], and are influenced by E toxicity [28], a proposed mechanism for the loss of cells expressing ER [28–30]. Regardless of these potential confounding factors, CR's attenuation of these age-related changes is unequivocal.

In light of the MPOA's critical role regulating reproductive function, CR's maintenance of MPOA cells and their sensitivity to estrogen has expected functional repercussions. Evidence that CR retards the onset of acyclicity [14] and maintains reproductive potential after a return to ad-libitum feeding [31], has been well documented. This delay of reproductive senescence can be explained by CR's maintenance of regulatory cells in the hypothalamic nuclei sensitive to estrogen. Of these nuclei, the pre-optic nucleus demonstrates the most estrogen sensitivity [32]. As such, CR's maintenance of MPOA cell populations explains these effects.

While reproductive senescence is not aging per se, it serves as an excellent model for studying whole organism aging. The observation that the maximum reproductive life span in most species of mammals is about half the maximum life span, suggests a strong link between mechanisms controlling reproductive life span and mechanisms controlling longevity. In addition, studies have shown that extension of the reproductive span appears to be the most important determinant of life span extension [33]. Some have even proposed that the beneficial effects of caloric restriction on longevity evolved due to the selective advantage for females whose reproductive life spans were extended by food restriction [34]. All this suggests that targeting the reproductive life span for extension may be one of the mechanisms by which caloric restriction extends the whole organism life span.

Although the mechanism by which CR extends the lifespan has yet to be determined, this work contributes to the growing body of evidence that:

1) Endocrine sensitivity in the hypothalamus changes throughout the life span.
2) The pattern of age-related change in the hypothalamus is predictable and sequential.
3) Caloric restriction delays or decreases the rate of normal age-related changes in the hypothalamus.

Assuming these observations are accurate, and accepting aging as a whole-organism phenomenon, a fundamental question remains. Are changes in the neuroendocrine hypothalamus a secondary phenotypic response, like that of other organ systems, to the underlying process of aging; or given its superior position in the hierarchy of whole organism influence, are changes in the hypothalamus driving the age-related phenotypic changes seen in other organ systems? [35, 36]. In other words, do changes in the neuroendocrine system regulate the rate of whole organism aging?

Mapping hypothalamic nuclei at specific times in the mammalian life span and tracking changes in cell numbers and hormone sensitivity with age and under the influence of caloric restriction will help establish neuroendocrine biomarkers associated with youthful phenotypes. With this knowledge, interventional studies can be designed to determine causality between maintaining these hypothalamic nuclei and slowing the rate of aging.

Acknowledgements

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