

Middle-aged female rats lack changes in histone H3 acetylation in the anterior hypothalamus observed in young females on the day of a luteinizing hormone surge

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Summary

Histone acetylation has recently been implicated in gene transcription and estradiol (E2) actions in the hypothalamus. This study aims to determine the involvement of histone acetylation in mediating E2-induced luteinizing hormone (LH) surge to understand the mechanism underlying LH surge dysfunction in female reproductive aging. Young and middle-aged female rats were ovariectomized (OVX) and treated with hormone or oil once per day for two days. At the time of the expected LH surge, blood samples were taken for LH assay. The anterior and posterior hypothalami were dissected, histone H3/H4 acetylation and histone deacetylases (HDACs) 4, -5, -10 and -11 protein expressions were measured using Western blotting. Our results show that in the young females, E2 markedly increased histone H3 acetylation while significantly reducing HDAC10 protein expression in the anterior hypothalamus. Notably, E2-induced alterations of histone H3 acetylation and HDAC10 in the anterior hypothalamus were absent in middle-aged females, associated with a reduced LH release. However, age alters histone H4 acetylation in both the anterior and posterior hypothalamus and significantly increased HDAC 4 and -5 protein expression in the anterior hypothalamus. Taken together, these data suggest that histone H3 acetylation in the anterior hypothalamus may mediate E2 regulation of LH surge and the process possibly through decreasing HDAC10. The missed responsiveness of histone H3 acetylation and HDAC10 expression to E2 in the anterior hypothalamus may contribute to LH surge failure that occurs in female reproductive aging.

Keywords: Histone acetylation, histone deacetylases, LH, hypothalamus, aging

1. Introduction

A robust and appropriately timed preovulatory luteinizing hormone (LH) surge requires an estradiol (E2) target on estrogen receptor alpha (ER α)-expressing

neurons in the hypothalamus to initiate gene transcription and the ensuing coordinated actions of these gene products on gonadotropin-releasing hormone (GnRH) neurons (1-4). As female rodents enter middle age, there is a characteristic delayed and attenuated LH surge under an E2 positive feedback condition, which has been considered as the earliest biomarker associated with reproductive aging (5-7). The neural and molecular mechanisms that underlie the age-related LH surge impairment remain incompletely characterized (6-9), the reduced LH surge is not induced by processes affecting the existence of E2-responsive cells (e.g. apoptosis) in the hypothalamus (8,10), but rather by mechanisms affecting the transcriptional activity of the network of

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genes related to neurotransmitter/neuropeptide release and recycling (9,11,12) in the anterior hypothalamus including the preoptic area (POA) and anteroventral periventricular nucleus (AVPV).

At the time of LH surge, what ultimately causes the decreased transcriptional activity of genes in the middle-aged hypothalamus remains poorly understood. Epigenetic changes such as histone acetylation can be induced by sex steroids (13,14) and have recently been implicated in the sexual differentiation of several brain (15,16) and behavioral phenotypes (13,15,17). Once the amino acid residues on the histone tail are acetylated, the bond between histones and DNA will be relaxed to allow access to transcriptional factors (18-21). Of great interest, histone acetylation is required for ER α -mediated gene transcription by affecting ER α to bind estrogen response element (ERE) within the regulatory regions of target genes (13,22). In the ventromedial nucleus of hypothalamus (VMH) and POA of female mice, histone H3 and H4 were highly acetylated following E2 administration, accompanied by transcriptional activation of the ER α target gene *Pgr* (13) as well as expression of reproductive behavior (23). Under E2 positive feedback conditions, histone H3K9/14 acetylation was induced by E2 to recruit ER α binding in the *Kiss1* promoter region in the AVPV and was positively associated with an increase in *Kiss1* gene expression in the nucleus (24). These studies indicate that histone acetylation facilitates ER α -mediated gene transcription to coordinate E2 actions in the hypothalamus. Typically, histone acetylation status is regulated by a variety of histone deacetylases (HDACs) (25-27). E2 induced histone H3 acetylation at the *Bdnf* promoter pII and pIV enhancing memory consolidation in the hippocampus is coupled with decreased HDAC2 and -3 (28), supporting the critical role of HDACs for E2 modulation of histone acetylation.

Neuroendocrine epigenetic modifications induced by aging and hormone cues have increasingly been suggested as good candidates to provide a molecular explanation for aging-related changes of brain function (29,30). In middle-aged mice, impaired spatial and contextual memory is associated with deficits in learning-induced H4 acetylation in the hippocampus (31,32); when E2 is administered in middle-aged mice, there is a specific increase of histone H3 acetylation at *Bdnf* promoters pI and pIV in the dorsal hippocampus (28). These studies implied that interaction of age and hormones could alter histone acetylation level in the brain to induce memory impairment. Thus, it is a perspective that attenuated histone acetylation in the hypothalamus under E2 positive feedback may be the underlying molecular mechanism of reduced transcription of genes in the hypothalamus and contributes to the impaired LH surge in middle-aged females.

The current study was designed to investigate the

potential age-related alteration of histone acetylation in the hypothalamus with regard to reduced E2-induced LH surge. We compared young and middle-aged female rats that were treated with a similar exogenous estradiol regimen known to elicit LH surges and determined *i*) whether hypothalamic histone acetylation level exhibits changes coincident with E2-induced LH surge, if so, does this occur similarly in different ages; *ii*) whether age or E2-related hypothalamic HDACs expression exists that may account, in part, for differential histone acetylation in middle-aged females during the LH surge.

2. Materials and Methods

2.1. Animal care

All animal procedures were approved by the Institutional Animal Care and Use Committee at Fudan University. Young (3-4 mo) and middle-aged (9-12 mo, retired breeders) female Sprague Dawley rats (Vital River, Beijing, China) were housed in groups and maintained on a 12-hour light, 12-hour dark cycle (lights on at 0700) with free access to chow and water. Rats were handled for 5 min/d for 1 week before monitoring estrous cyclicity by daily vaginal lavage with sterile saline for 2 weeks. Only rats with at least two regular 4- to 5-day estrous cycles were included in the studies (5,8,9).

2.2. Ovariectomy surgery and hormone administration

Eighteen young and eighteen middle-aged rats were anesthetized with pentobarbital sodium (30mg/kg, *ip*) and ovariectomized (OVX). To induce LH surges, females received 2 μ g/0.1ml subcutaneous injections of estradiol benzoate (E2; Hangxiang Inc., China) dissolved in peanut oil. At 0900h on day seven after OVX, rats received the first of two daily injections of 2 μ g of E2. For the temporal LH surge study, forty-eight hours after the first E2 injection, rats were injected with 500 μ g of progesterone (P; Steroloids, Inc.). This hormone regimen reliably produces LH surges in female rats (5,8,9).

2.3. Jugular vein catheterization and blood collection

On postoperative day seven rats were anesthetized and an indwelling catheter placed into the right atrium via the right jugular vein for serial blood sampling (5). Catheters were kept patent with daily heparinized saline flushes. Serial blood samples (300 μ L) were collected from awake, freely moving rats for determination of the LH surge at 1300h, 1500h, 1700h and 1900h. Each sample volume was replaced with warm, sterile, heparinized saline (15 U/mL). Plasma was stored at -80°C until LH determination.

2.4. Hypothalamic dissection

At the time of peak LH release, young and middle-aged rats were euthanized by rapid decapitation for Western blotting experiments. As previously described (5,8,33), the anterior hypothalamus, which includes the POA, and the posterior hypothalamus, which includes the arcuate and VMH, were flash frozen on dry ice, and stored at -80°C until quantitation of proteins.

2.5. Western blotting

Samples were resuspended and sonicated in hypotonic lysis buffer (34). Total protein content of the lysates was measured using BCA protein assay (Thermo Scientific), sample buffer was added, and samples were boiled for 5 min at 100°C. Samples were loaded onto Tris-HCl polyacrylamide gels (Bio-Rad) for electrophoresis and, after separation, transferred to PVDF membranes (Millipore). Membranes were incubated overnight at 4°C with rabbit polyclonal antibodies recognizing histone H3 (1:1000, Abcam), acetylated histone H3 (1:5000, Millipore), histone H4 (1:1000, Abcam), acetylated histone H4 (1:1000, Millipore), HDAC4 (1:1000, Santa Cruz Bio), HDAC5 (1:1000, Santa Cruz Bio), HDAC10 (1:1000, Santa Cruz Bio), HDAC11 (1:1000, Santa Cruz Bio), or monoclonal β -Actin (1:5000, Sigma). After TTBS wash, membranes were incubated for 1h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) or with goat anti-mouse IgG (Sigma Aldrich) and developed using enhanced chemiluminescence (SuperSignal West Dura, Thermo Scientific). Signal was detected using a Kodak Image Station 440CF and quantified by densitometry using Kodak 1D 3.6 software.

2.6. LH assay

Serum LH concentrations were measured using enzyme-linked immunosorbent assay (ELISA) with LH

reagents provided by the Beijing Sino-UK Institute of Biological Technology (Chaoyang, Beijing).

2.7. Data analysis

GraphPad Prism 6 software was used for statistical analysis. Data are expressed as mean \pm SEM. The area under the curve (AUC) for total LH release was calculated using Sigma Plot 10.0; *t*-test was used to determine difference in total LH release. Two-way ANOVA (age \times hormone) was utilized to detect differences in histone acetylation and HDACs protein expression in anterior and posterior hypothalamus in young and middle-aged groups. Bonferroni or Turkey's *post-hoc* tests were performed to determine individual group differences following main or interaction ANOVA effects.

3. Results

3.1. Middle-aged rats exhibit an attenuated LH release under E2 positive feedback

We first sought to replicate our previous results that E2-induced a reduced LH surge in middle-aged rats under E2 positive feedback. The middle-aged rats exhibited an obvious decreased LH surge after the first E2 injection (Figure 1A). When compared with the middle-aged rats, young rats released 3-fold as much LH ($p < 0.01$) at the time of E2-induced LH surge (Figure 1B). These results are in agreement with our previous studies and others (5-7).

3.2. E2-induced histone H3 acetylation was absent in the anterior hypothalamus of middle-aged rats

We next determined whether E2-induced LH surge is associated with hypothalamic histone acetylation, and if so, whether histone acetylation in the hypothalamus is altered in middle-aged females. Total extracts of anterior and posterior hypothalamus were isolated

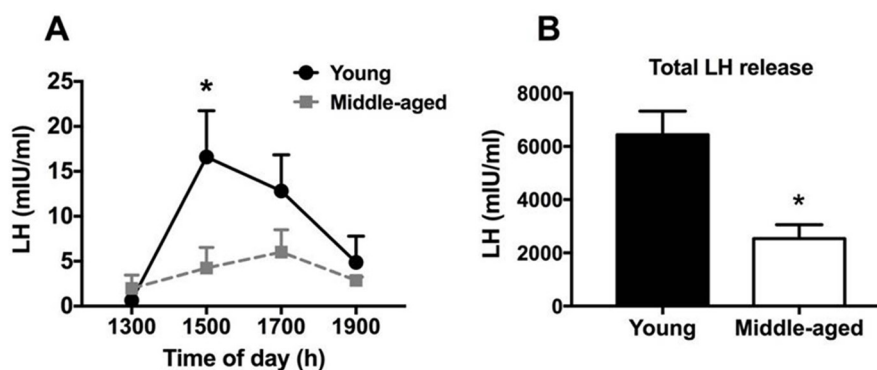


Figure 1. Hormone-induced LH surge was reduced in middle-aged female rats. OVX young and middle-aged rats were primed with two daily doses of E2 benzoate (2 μ g) and one injection of P (500 μ g) on the day of blood collection. (A) LH surge and (B) Total LH release in young and middle-aged females. Data are shown as means \pm SEM, $n = 6$ in both groups, * $p < 0.01$.

from E2 and vehicle-treated young and middle-aged rats during the LH surge, effects of age and hormone treatment on the global acetylation levels of histone H3 and H4 (H3Ac and H4Ac) were analyzed by Western blotting. In the anterior hypothalamus, a significant main effect of E2 ($F = 12.81$, $p < 0.01$) was found for acetylated histone H3, higher in E2 than vehicle-treated young females (Figure 2A). An interaction between age and E2 on H3Ac ($F = 5.14$, $p < 0.05$) was observed as well; E2-induced a higher acetylated histone H3 level in young females, however, this effect was absent in middle-aged rats (Figure 2A). Interestingly, there was no significant main effect of age, or any significant hormone by age interactions, for histone H3 acetylation in the posterior hypothalamus (Figure 2B).

3.3. Age but not E2 alters histone H4 acetylation in the anterior or posterior hypothalamus

However, regardless of E2 treatment, a significant main effect of age was found for acetylation of H4 in the anterior hypothalamus ($F = 7.398$, $p < 0.05$) (Figure 3A), with acetyl H4 level higher in middle-aged compared to young females. A significant main effect of age on H4 acetylation was also found in the posterior hypothalamus ($F = 7.398$, $p < 0.01$) (Figure 3B), however, the H4 acetylation level was lower in middle-aged compared to young females. These results implied that the effect of E2 on histone acetylation in the hypothalamus is specific to H3.

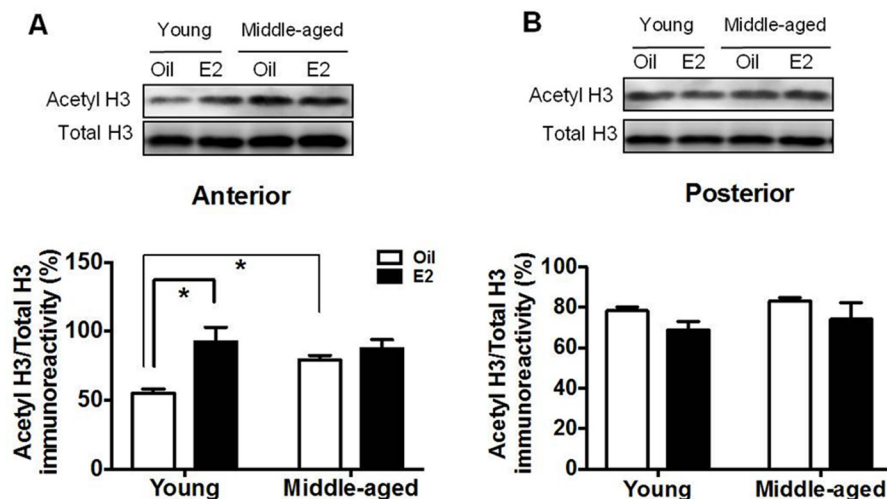


Figure 2. Effect of E2 on histone H3 acetylation in the anterior hypothalamus of young and middle-aged female rats at the time of LH surge. Rats were given two daily doses of E2 benzoate (2 μ g) and were killed 52-54 hrs after the first injection of E2. (A) Western blot showing E2 increases histone H3 acetylation level in the anterior hypothalamus of young but not middle-aged females. (B) E2 does not change histone H3 acetylation in the posterior hypothalamus of young and middle-aged rats. Data are shown as means \pm SEM, $n = 6$ in both groups, * $p < 0.01$.

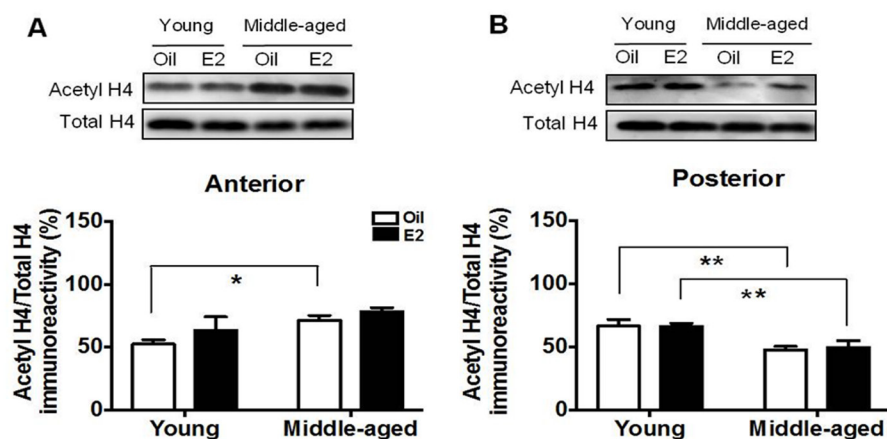


Figure 3. Age alters histone H4 acetylation in the anterior and posterior hypothalamus of young and middle-aged rats. Independent of hormone priming age affects histone H4 acetylation in the anterior and posterior hypothalamus. Rats were given two daily doses of E2 benzoate (2 μ g) and were killed 52-54 hrs after the first injection of E2. (A) Middle-aged females show an increased histone H4 acetylation in the anterior hypothalamus. (B) Acetylated histone H4 decreases in the posterior hypothalamus of middle-aged females. Data are shown as means \pm SEM, $n = 6$ in both groups, * $p < 0.05$, ** $p < 0.01$.

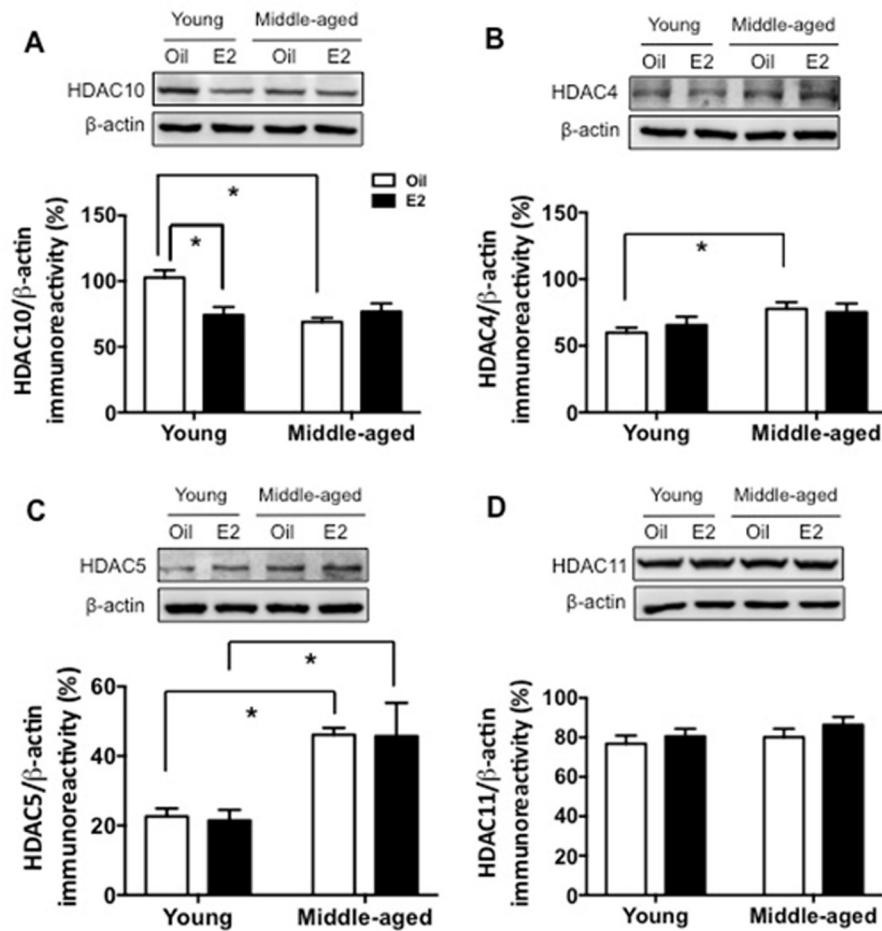


Figure 4. E2 decreases HDAC10 protein expression in the anterior hypothalamus of young but not middle-aged rats at the time of LH surge. Rats were given two daily doses of E2 benzoate (2 μ g) and were killed 54-56 hrs after the first injection of E2. Western blotting showing (A) E2 priming decreases HDAC10 protein level in the anterior hypothalamus of young but not middle-aged females. (B) HDAC4, (C) HDAC5 and (D) HDAC11 protein expression in the anterior hypothalamus of young and middle-aged females. * $p < 0.05$.

3.4. E2 decreases level of histone deacetylase 10 in the anterior hypothalamus of young but not middle-aged rats

HDACs remove acetyl groups from histone tails, thereby condensing the chromatin and decreasing gene transcription (25,35). We previously showed that E2 treatment positively decreased mRNA levels of 4 HDACs (*Hdac4*, -5, -10 and -11) in the anterior hypothalamus of young females (36). However, E2 does not change *Hdac4*, -5, and -11 mRNA expression in middle-aged females except *Hdac10* (36). The current Western blotting found that there was an interaction between E2 and age on HDAC10 protein expression in the anterior hypothalamus ($F = 6.063$, $p < 0.05$) (Figure 4A). Although HDAC10 decreased in young rats following E2 treatment, this effect was absent in middle-aged rats. Regardless of E2 treatment, HDAC4 ($F = 5.435$, $p < 0.05$) and HDAC5 ($F = 8.293$, $p < 0.05$) protein was higher in middle-aged compared to young rats (Figure 4B and 4C). There was no significant main effect of age, or any significant hormone by age interactions, for HDAC11 in the anterior hypothalamus (Figure 4D).

4. Discussion

Although sex steroid induction of histone acetylation has been recognized as an important mechanism mediating sexual differentiation of brain (15,16) and behavioral phenotypes (13,15,17), the possibility that histone acetylation participates in steroid-induced LH surge remains largely unknown. The present experiments demonstrate for the first time that histone H3 acetylation in the anterior hypothalamus is enhanced by E2 at the time of LH surge. Specifically, E2 induced increased histone H3 acetylation may causally be linked to reduced protein expression of HDAC10 in the anterior hypothalamus. Notably, the E2-administration, which led to a significant increase in the level of histone H3 acetylation is absent in the middle-aged anterior hypothalamus, coincident with the typical impaired E2-dependent LH surge. To our knowledge, this is the first evidence demonstrating that E2-induced increased histone acetylation especially H3 in the anterior hypothalamus may be involved in LH surge, which is partially consistent with a recent report indicating that E2 administration led to a significant change in histone H3

acetylation in the *Pgr* gene in the POA of female mice (13). Thus, these data suggest that the missed E2-induced histone H3 acetylation in the anterior hypothalamus may be an important mechanistic pathway for the changes of E2 target genes expression and consequently results in age-related impaired LH surge in middle-aged females. However, histone H4 acetylation, unlike histone H3, showed mainly age-related changes in the anterior and posterior hypothalamus.

Acetylation of histone proteins has long been known to promote transcriptional activity (21,37) and contribute to E2-induced gene expression in human MCF-7 cells (14,38,39) and specific hypothalamic regions (13,40,41). Of great interest, histone acetylation is crucial in E2-mediated sexual behavior (13) and formation of hippocampus-dependent memory in mice (28,34,42,43). We therefore tested the hypothesis that histone acetylation in the hypothalamus would be regulated by E2 to generate a GnRH/LH surge in young females. We observed distinct elevated histone H3 acetylation detected at the level of global chromatin in the anterior hypothalamus in response to E2 at the time of LH surge, suggesting histone hyperacetylation induced by E2 is able to facilitate E2-induced LH surge in female rats. Acetylation of Lys residues on histone H3 and H4 was shown to be critical for an open chromatin structure and gene transcription (35,38), especially acetylation of H3K56ac and H3K14ac is associated with expression of genes by regulating genome stability (35). However, the current data is limited to reveal the E2-induced specific acetylation marks on histone H3 during LH surge. On the other hand, LH surge requires E2 stimulating appropriate induction of ER α to bind ERE (14) within the regulatory regions of target genes in glutamatergic and GABAergic neurons in the POA (6,7), histone H3K9/14 acetylation and ER α binding in the AVPV *Kiss1* promoter region were induced by E2 and were positively associated with an increase in *Kiss1* gene expression in this nucleus (24). Future studies should specifically examine which genes in those specific cell types will be regulated by histone H3 acetylation to gain a better understanding of how E2 regulates gene transcription for a GnRH-LH surge.

HDACs are recognized as important mediators of epigenetic changes via histone deacetylation and chromatin remodeling (25,27). Thus, decreased HDACs should alter histone acetylation and open up chromatin, allowing for increased gene expression. Our data show here that female rats with E2-induced LH surge have enhanced histone H3 acetylation in the anterior hypothalamus but a decrease in HDAC10. HDAC10 was found to be expressed in neurons (44), fasting decreased HDAC10 in the medial basal hypothalamus (45). Our finding that HDAC10 in the anterior hypothalamus decreased at the LH surge time imply that HDAC10 may play a role as a crucial negative regulator of LH surge.

One of the important observations from our study was that E2-regulated histone H3 acetylation is absent

in the anterior hypothalamus of middle-aged females at the LH surge time. Age-related changes in the LH surge mechanism are recognized as a reflection of reduced gene transcriptional activity of GnRH neuron excitatory afferent inputs in the POA (8,9,12). By analyzing histone acetylation levels in the anterior hypothalamus, we could speculate that a loss of histone H3 acetylation responses to E2 may disrupt the induction of the ER α -mediated gene program in the POA. Nevertheless, it should be noted that we did not use the restricted POA tissues for CHIP experiments, defining the specific targets of the acetylated histone H3 and the putative gene promoters is of paramount importance. Our data showed that middle-aged females lacking E2-induced histone H3 acetylation in the anterior hypothalamus have a consistent unchanged level of HDAC10. The missed response of HDAC10 to E2 could directly link with histone deacetylation and consequently decrease gene transcription by condensing chromatin.

Recent evidence suggests that epigenomic changes can occur extremely early in the aging process and be causative. Hypothalamus-mediated aging may contribute to physiological deterioration and aging-related disease (29,46). However, it is largely unknown whether these aging-associated changes are a cause or a consequence of histone acetylation. Interestingly, we found that age changes histone H4 acetylation in both the anterior and posterior hypothalamus and HDAC4 and -5 protein expression in the anterior hypothalamus. Histone H4 modification such as H4K20me3 has been demonstrated to be altered during mammalian aging (47), and an increase in H4K20me3 was found in human patients with Hutchinson-Gilford progeria, a premature aging syndrome (48). This evidence implies hypothalamic acetylated histone H4 and specific HDACs may be involved in hypothalamus programming aging phenotypes including energy homeostasis or circadian rhythm.

In conclusion, the present study demonstrates histone H3 acetylation in the anterior hypothalamus as a novel mechanism underlying the E2 positive feedback action to induce GnRH-LH surges, and expression of HDAC10 in the anterior hypothalamus is regulated by E2. Our findings provide the first evidence that the middle-aged female anterior hypothalamus loses histone H3 acetylation and HDAC10 expression responsive to E2, and overall suggest an epigenetic mechanism may underlie reduced gene transcriptional activity and the associated LH surge dysfunction in middle-aged females. These findings may provide a key insight into the mechanism of age-related loss of responsiveness of the hypothalamus under E2 positive feedback.

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