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EXPOSURE TO ESTRADIOL IMPAIRS LUTEINIZING HORMONE FUNCTION DURING AGING

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SUMMARY

The loss of ovulatory cyclicity in many mammals is caused by changes in the hypothalamic-pituitary (H-P) control of the preovulatory luteinizing hormone (LH) surge. This work evaluated the anterior pituitary (AP) component of the H-P axis by determining the ability of perifused AP to release LH following sustained but pulsatile LHRH stimulation. The normal dual discharge profile of LH was affected by age. The first hour of the response, unaffected by cycloheximide, was similar in 5—6-month-old (mature), 12—13-month-old (declining litters) and 16—18-month-old (irregularly cycling) mice. The remaining protein synthesis-dependent part of the response was reduced in the 16—18 and 22—24-month-old (anestrus) mice. The role of estradiol (E2) in AP aging was further tested as AP from ovariectomized (OVXed) mice, deprived of E2 since puberty, responded as well as the mature proestrous group. In contrast, aged mice subjected to long-term E2 exposure (cycling or OVXed plus E2 replacement) failed to produce the dual response pattern. Since alterations in LH response occurred during the protein-dependent phase, synthetic processes that involve packaging and transport of stored LH, or the production of new LH, may be affected by age. Futhermore, E2 is a major factor in altering LH function and appears to act before middle age.

Key words: Perifusion; Pituitary gland; Neuroendocrine aging; Luteinizing hormone; C57BL/6 female mouse

INTRODUCTION

The hypothalamic-pituitary (H-P) complex loses the ability to control ovulation by middle age in many laboratory animals. The gradual loss in cyclicity correlates

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to alterations in the preovulatory LH surge [1,2] that are independent of the aging process of the ovary, but dependent upon long-term exposure of the H-P to ovarian steroids, especially to estradiol (E2) [3—5]. Current research indicates that much of the neuroendocrine impairment lies centrally in the hypothalamic control over the anterior pituitary (AP) [6—8]. There is some evidence, however, that alterations in cyclicity may also involve inherent changes in AP function [4,9,10]. Due to the close anatomical and chemical link between the AP and hypothalamus, it is difficult to determine how each tissue contributes to the loss of LH function. To overcome this problem, an in vitro perifusion system to isolate the AP from the brain was used. The purpose of this study was to evaluate the effect of age on the ability of the perifused AP to release LH in response to sustained, but pulsatile LHRH stimulation. The role of E2 in AP aging was also evaluated through long- and short-term ovariectomy (OVX), with and without E2 replacement.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice were purchased or raised from mice obtained from Houston Sprague—Dawley Breeders (Houston, TX) and from Charles Rivers Breeders (Stone Ridge, NY). The mice were housed six per cage in mouse quarters (14L/10D; lights on 0600 h; 21°C) with food and water supplied ad libitum.

Tissue collection

On the day of the perifusions, the appropriate animals were killed by decapitation between 1100 and 1200 h. The pituitaries were rapidly removed, the neural lobe discarded and the AP quartered. Fragments equivalent to two pituitaries were then transferred to individual incubation chambers.

Perifusion

The AP preparations were perifused as described previously [11] in incubation chambers constructed from 1-ml tuberculin syringes. After a 2-h equilibration period of Medium-199 flow alone (M-199; 5 ml/h), the preparations were challenged with M-199 plus LHRH. Ten-minute pulses of 10 nM LHRH at 20-min intervals for 6 h causes LH release in two distinct episodes when AP of mature animals (5—6-month-old) are perifused [11]. Ten chambers were perifused simultaneously and representative groups from each study were perifused on separate days to randomize the data collection. The individual effluents were collected at 10-min intervals and stored frozen in duplicate 100-μl volumes until assayed for LH content using NIADDK rat LH RIA kits [12].

Studies

Study No. 1. To determine the effect of age on LH function, AP from mice in four

reproductive stages: reproductive prime (5—6-month-old), reproductive decline (12—13-month-old), irregularly cycling (16—18-month-old) and an anestrous state (22—24-month-old) were perifused. Two to four weeks prior to sacrifice daily vaginal smears were examined. Because the oldest group exhibited constant leukocytic smears, mice from the other groups were killed in the diestrous stage of their estrous cycle. The AP were perifused as described above.

Study No. 2. To judge the usefulness of OVX and steroid-priming (SP) on AP function, 5—6-month-old mice were OVXed or sham-OVXed at 3—4 months of age. Half of the OVXed group were SP with an E2 implant (0.28 g 17β-estradiol per g K-Y jelly in Clay Adams PE200 tubing; 1 mm per g body weight) for 6 days, followed by two additional implants for 1 day [13]. The other half received K-Y jelly filled implants. This E2 regime produced proestrous-like LH surges in young OVXed mice one day (1800—2100 h) after the addition of the two E2 implants. Sham-operated mice in proestrus and the two OVXed groups were killed and the AP perifused as described above.

Study No. 3. To determine the effect of E2 and age upon AP LH function, mice were OVXed at either 3, 8 or 16 months of age. Those OVXed at 3 months and 8 months were either given E2 (1 μg 17β-estradiol in 10 μl sesame oil) or vehicle weekly s.c. until 16 months of age. After a 2-month quiescent period, each group, plus a 5-month-old group OVXed 2 months earlier, were SP and perifused as described above.

Data handling

LH point values for each perifusion were converted to area under the curve, above baseline, for 1-h time intervals. For each study, the relative areas for each time interval, within and between the groups, was analyzed using a multi-factoral ANOVA for repeated measures with Newman-Keul's multiple range test applied to sort out differences [14]. The amount of LH released during periods 1, 2, 5 or 6 were considered to contribute to peak discharges if the amounts were significantly higher than LH releases during the intervening periods 3 or 4. The 7th release period represents the post-stimulus time period and was not used in determining peak discharges. Probabilities less than 0.05 were considered statistically significant. Each group was represented by 4—6 preparations.

RESULTS

Study No. 1

AP from 5—6-month-old and 12—13-month-old diestrous mice, subjected to 10 min, 10 nM LHRH pulses, released LH in two distinct episodes (Fig. 1; Table I) that peaked in the 1st and then in the 5th or 6th release periods. LH release from the other two groups exhibited one peak discharge that occurred only during the 1st release period. Although a second rise in LH release was found in the 15—16-month-

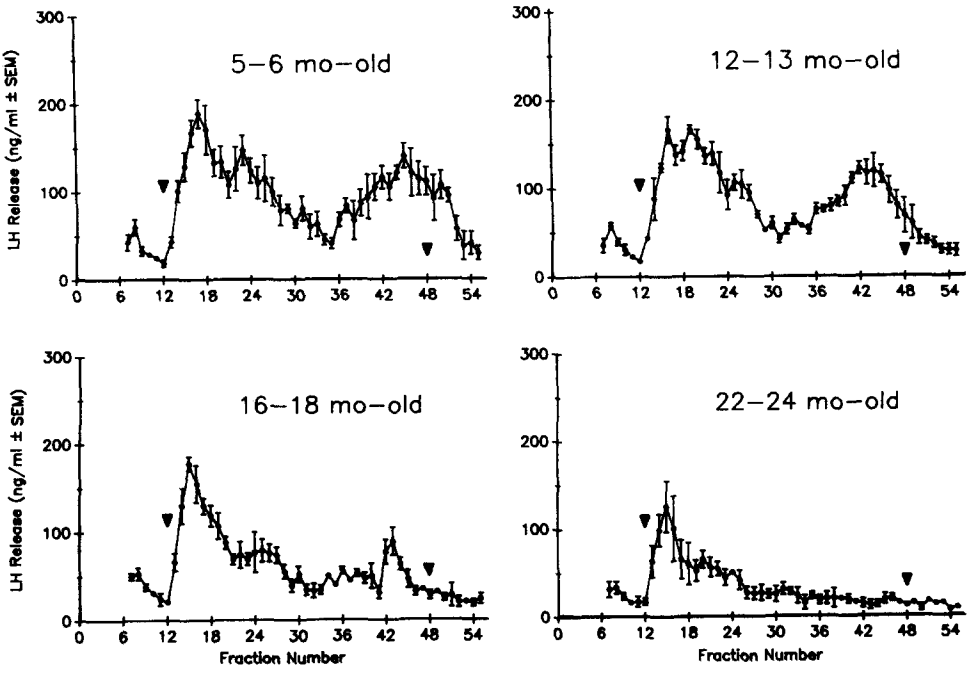

Fig. 1. A comparison of LH release from AP derived from female mice at four different reproductive stages: 5—6-month-old, mature; 12—13-month-old, reproductive decline; 16—18-month-old, irregularly cycling; 22—24-month-old, post-reproductive. Arrow heads indicate the start and end of 10 min, 10 nM LHRH pulses (3/h).

old group during period 5 that was significantly higher than releases in period 6, it was not significantly higher than releases in periods 3 or 4 and was therefore, not considered to contribute to a second discharge episode. A comparison of LH secretions during the first release period revealed that significantly less LH was released in the 22—24-month-old group than in the other age groups. Similarly, hourly LH releases for the two oldest groups during release periods 2—7 was significantly lower than releases in the two younger groups. The total amount of LHRH-induced LH release during the entire 7-h release periods was also progressively smaller as age increased when comparing the 5—6-month-old or 12—13-month-old groups with the 15—16-month-old and 22—24-month-old groups.

Study No. 2

Regardless of steroid manipulation, AP from young mice released significant amounts of LH in response to LHRH during the first hour of stimulation that then declined significantly during the second hour of stimulation in the proestrous and OVX non-SP groups (Fig. 2; Table II). Hourly releases from the OVX non-SP group

TABLE I

THE EFFECT OF AGE AND REPRODUCTIVE STATE ON LH RELEASE (AREA ± S.E.M.)

Months of age	*Hourly release periods*							*Total*
	1st	*2nd*	*3rd*	*4th*	*5th*	*6th*	*7th**	
5—6	398 ± 30[a]	317 ± 31[cdh]	202 ± 30[ah]	122 ± 18[ai]	229 ± 23[cfh]	291 ± 22[aj]	126 ± 25[i]	1686 ± 127[h]
12—13	376 ± 12[a]	324 ± 27[ah]	172 ± 24[fh]	141 ± 34[fi]	255 ± 20[bcfh]	221 ± 31[cfj]	58 ± 14[j]	1545 ± 062[h]
15—16	377 ± 16[a]	170 ± 33[ai]	110 ± 16[efi]	73 ± 28[f]	111 ± 28[efi]	62 ± 06[fi]	12 ± 03[g]	922 ± 044[i]
22—24	238 ± 24[ag]	121 ± 15[ag]	47 ± 17[defg]	30 ± 06[f]	18 ± 05[g]	13 ± 07[g]	8 ± 04	468 ± 048[g]

*Post-stimulus release period.

Significant within-age group differences (across): [a]Higher than all lower release periods. Higher than [b]3rd, [c]4th, [d]5th, [e]6th, [f]7th release periods.

Significant between-age group differences for the same release period (down): [g]Lower than all higher releases. [h]Higher than the two lowest releases. [i]Higher than the lowest release. [j]Higher than all lower releases.

TABLE II

THE EFFECT OF OVARIECTOMY (OVX) AND STEROID-PRIMING (SP) ON LH RELEASE (AREA ± S.E.M.)

Group	*Hourly release periods*							*Total*
	1st	*2nd*	*3rd*	*4th*	*5th*	*6th*	*7th**	
Proestrus	445 ± 014[ai]	311 ± 009[cdg]	191 ± 07[ai]	163 ± 12[gi]	306 ± 017[cdg]	302 ± 018[a]	111 ± 13[i]	1829 ± 057[i]
OVX non-SP	382 ± 056[b]	196 ± 042[i]	279 ± 43	465 ± 92[b]	461 ± 082[b]	498 ± 106[b]	467 ± 81[b]	2748 ± 344
OVX-SP	775 ± 119[dgh]	566 ± 140[h]	506 ± 43[h]	447 ± 35	958 ± 141[bcdgh]	864 ± 106[cdeh]	463 ± 80	4579 ± 526[h]

*Post-stimulus release period.

Significant within-age group differences (across): [a]Higher than all other lower release periods. Higher than [b]2nd, [c]3rd, [d]4th, [e]5th, [f]6th, [g]7th release period.

Significant between-age group differences for the same release period (down): [h]Higher than other releases. [i]Lower than all higher releases.

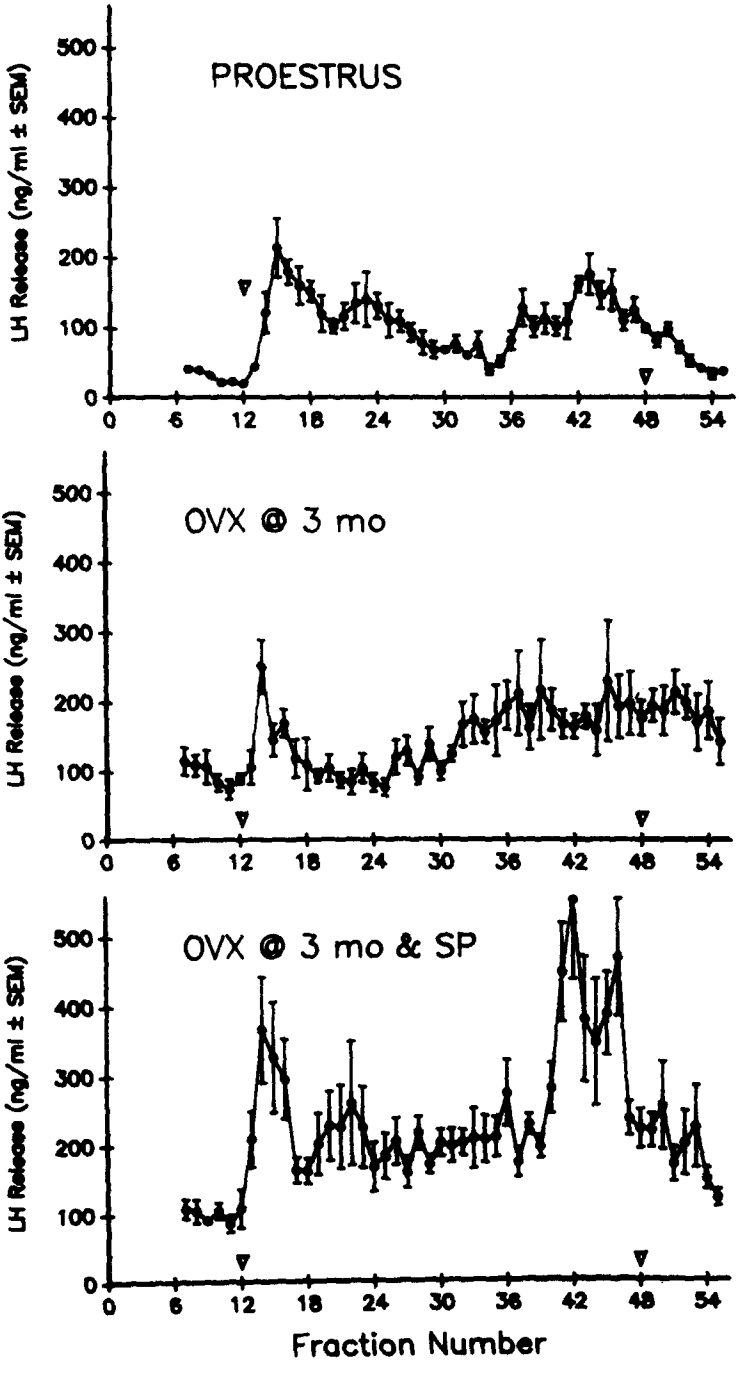

Fig. 2. Comparison of LH profiles of AP derived from 5—6-month-old female mice following sham-OVX (proestrus), OVX or OVX plus steroid priming (SP). Surgeries were performed at 3 months of age. Arrow heads indicate the start and the end of 10 min, 10 nM LHRH pulses (3/h).

grew progressively higher from periods 2 to 4, then plateaued and remained high after the stimulus was removed. In contrast, LH release from AP of the OVX-SP group and the proestrous group exhibited more typical dual discharge patterns that quickly dropped to significantly lower levels during the post-stimulus release period. The AP from young OVX non-SP mice, released 2.4 times more LH overall, however, than the proestrous group. Similarly, total LH release from the OVX-SP group was 1.7 times that of the OVX non-SP group.

Study No. 3

When the combination of age and E2 manipulations was studied (Fig. 3; Table III), AP from 18-month-old mice OVXed at 3 months, without E2 replacement (group 5), released less total LH than the young OVX-SP group (group 1), although

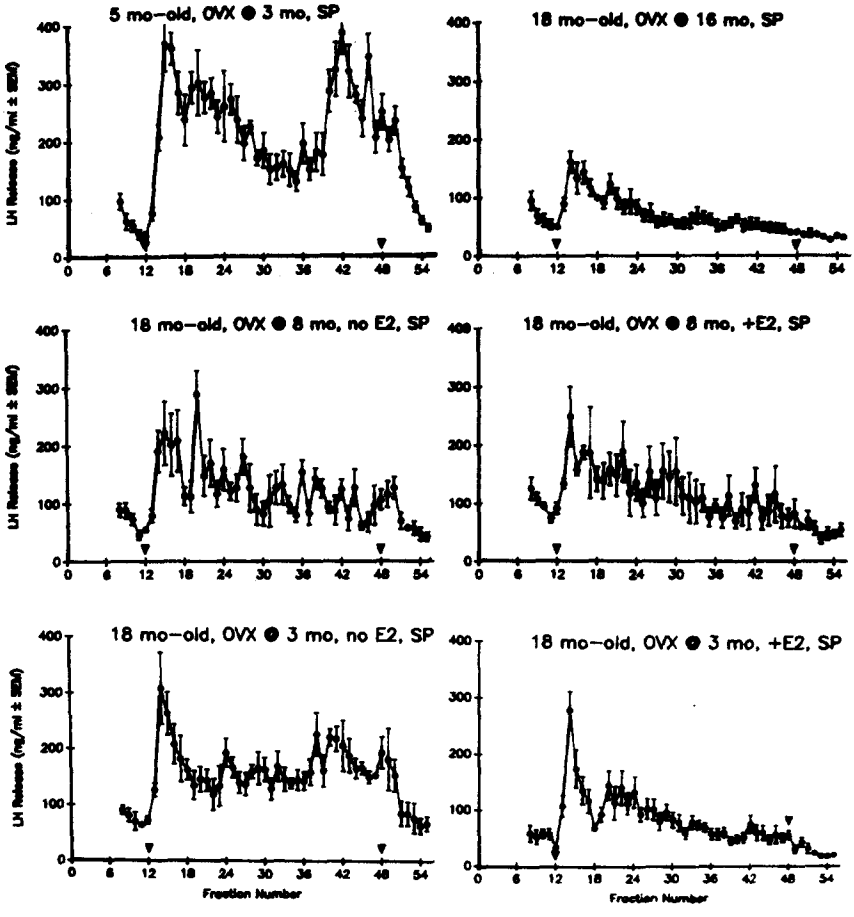

Fig. 3. The effect of OVX timing and E2 replacement on LH release from perifused AP derived from 18-month-old steroid-primed female mice. A 5-month-old OVX-SP group was included as a control (upper-left panel). The arrow heads indicate the start and end of 10 min, 10 nM LHRH pulses (3/h).

TABLE III

THE EFFECT OF AGE AND E2 MANIPULATION ON LH RELEASE (AREA ± S.E.M.)

Group	*Months of age*		*Hourly release periods*							*Total*
	OVX	*Kill*	*1st*	*2nd*	*3rd*	*4th*	*5th*	*6th*	*7th**	
1	3 (N)	5	738 ± 49^{bg}	710 ± 95^{bf}	500 ± 60^{cg}	370 ± 28^{f}	729 ± 77^{bf}	651 ± 51^{f}	246 ± 25^{g}	3944 ± 310^{f}
2	16 (N)	18	340 ± 30^{abd}	211 ± 32^{bd}	118 ± 18	109 ± 20	97 ± 17	63 ± 13	37 ± 06	975 ± 099
3	8 (O)	18	439 ± 14^{bdh}	396 ± 53^{bdh}	250 ± 76	232 ± 23^{h}	218 ± 17^{h}	185 ± 35^{g}	94 ± 09^{f}	2061 ± 181^{g}
4	8 (E)	18	470 ± 47^{abdh}	316 ± 51^{d}	308 ± 73^{d}	176 ± 38	172 ± 37	151 ± 26^{g}	51 ± 11^{g}	1634 ± 240^{g}
5	3 (O)	18	586 ± 34^{abeg}	376 ± 40^{h}	384 ± 39^{h}	356 ± 41^{g}	567 ± 44^{abf}	422 ± 45^{f}	194 ± 51^{f}	2884 ± 115^{f}
6	3 (E)	18	409 ± 42^{bd}	321 ± 60^{cd}	221 ± 46^{e}	160 ± 28	122 ± 28	100 ± 21	30 ± 09	1362 ± 209

Significant within-group differences (across): *Post-stimulus period; LH releases all different from other hourly releases. Higher than [a]2nd, [b]3rd and 4th, [c]4th, [d]5th and 6th, and [e]6th hourly releases.

Significant between-group differences for the same release period (down): [f]Higher than all lower releases. [g]Higher than lower releases, except for the immediate lower release. [h]Higher than lowest release only.

Letters in parenthesis indicate weekly injections after OVX of either E2 (E) or oil only (O), or no injections (N). Injections ended 2 months prior to kill age.

a dual release pattern was found in both. In contrast, AP from 18-month-old mice OVXed at 16 months (group 2), or at 8 months (group 4) or at 3 months (group 6) and given E2 weekly until 16 months of age, did not produce dual LH discharge patterns and were unable to sustain elevated releases of LH past the first hour of stimulation. Hourly LH responses from group 4 were consistently higher than group 2, although the differences reached significant levels only during the 1st release period. The amount and pattern of LH releases were similar in aged mice OVXed at 8 months of age, regardless of E2 replacement (group 3 vs. group 4). In comparison, LH responses in aged mice OVXed at 3 months were consistently lower in animals receiving E2 replacement (group 6) compared to oil-treated animals (group 5). The differences were not significant in periods 2 and 3, however.

DISCUSSION

The production of the dual discharge of LH from perifused AP appears to be an excellent tool for studying mechanisms involved in the sustained release of LH. The first hour of release likely reflects the size of the readily-releasable LH pool since the release of this pool is not dependent upon immediate protein synthesis [11,15,16]. The remainder of the first discharge, significantly reduced by the protein synthesis inhibitor cycloheximide, is dependent upon protein synthesis of some type. Since newly synthesized LH is not a significant contributor to LHRH-induced LH release until approx. 3 h after sustained LHRH stimulation [17], the remainder of the first response likely depends upon the transport and conversion of stored LH to the releasable form [13,16]. Both processes rely on protein synthesis, but not on the synthesis of new LH. The second release peak occurs 4—6 h after the first episodic release and likely involves both newly synthesized LH and the conversion of stored LH to the releasable form [18].

In study No. 1, LH release in the two older groups was significantly affected during the protein synthesis-dependent part of the response. By 16 months of age, C57BL/6 female mice cycle irregularly, and exhibit attenuated or lost capacity for producing preovulatory LH surges. Because the amount of LH released during the first hour of LHRH stimulation was not affected at 16 months of age, it appears that altered LH function can be attributed, in part, to mechanisms that promote the sustained release of LH past the first hour of stimulation. The site of impairment could lie in the mobilization of stored LH since the AP of 16-month-old mice contain twice as much LH as those of young mature mice [19]. The AP of the 22—24-month-old group suffers from an even greater impairment. AP from this age group also contain significantly more LH than those of young mice, but the amount that is releasable in the first hour of stimulation was also significantly reduced. This confirms previous studies where smaller releases of LH were found in aged mice given single LHRH injections [12,20] or in pituitary cultures given hourly pulses of LHRH [10].

LH function in female animals is often assessed by studying the preovulatory LH

surge, however, C57BL/6NNia mice over 16 months of age rarely cycle, or cycle so infrequently, that finding sufficient aged animals in a preovulatory state, at any given time, is difficult. Aged mice in this strain exhibit long periods of leukocytic smears (constant diestrus, CD), seldom entering states of constant estrous or persistent pseudopregnancy as most aged female rats and mice. The 5—6 and 12—13-month-old mice in this study were, therefore, killed in diestrus so that their vaginal smears matched those of the older groups. It was not surprising that AP from CD and anestrous (ANE) animals respond poorly to LHRH stimulation in comparison to those from cycling diestrous animals. AP from diestrous animals are highly receptive to LHRH stimulation and exhibit similar levels of LHRH receptors [21], LH stores and the amount of readily-releasable LH pools as AP from proestrous mice [22,23]. Regardless, the data presented here adds to our knowledge by suggesting that specific mechanisms may be altered with age in AP from animals in different, but unmanipulated reproductive states.

To specifically test for neuroendocrine impairments, transplantations have been performed between host and donor mice of different ages [5,24,25]. In addition, the induction of proestrous-like LH surges in OVXed mice primed with various regimes of ovarian steroids has been an effective tool [5,25—27]. Such studies, however, indicate that neuroendocrine impairments exist without implicating the specific site or degree of involvment. We, therefore, sought a protocol to complement our perifusion technique that would subject the H-P complex of all age groups to identical, and normally facilitory, ovarian steroid environments. The protocol of Mobbs et al. (1984) proved to be ideal. The technique combined OVX and a two step E2 regime to induce preovulatory-like LH surges in young female mice, and to some degree in previously non-cycling aged mice. Our test of the protocol in combination with AP perifusions on young animals was presented in study No. 2. In practice, the protocol was not used to induce LH surges in vivo, but to prime the AP for in vitro LHRH stimulation. The E2 regime proved quite dependable. The priming E2 implants resulted in plasma E2 levels of 9 ± 2 pg/ml on day 6, while the surge producing E2 implants resulted in plasma E2 levels in the preovulatory range (43 ± 11 pg/ml) and vaginal smears composed primarily of rounded epithelial cells. In OVXed animals receiving only K-Y jelly implants, plasma E2 levels were near the level of sensitivity of the assay (3 ± 1 pg/ml) and resulted in predominately leukocytic vaginal smears.

Our perifusion results using AP from young OVXed mice generally agrees with in vivo studies [28] by demonstating the facilitory effects of OVX on LH secretion. Futhermore, we showed that total LH response was significantly greater in OVX-SP than in OVX non-SP mice. Even though AP from OVX-SP mice gave greater responses to LHRH than AP from proestrous mice, the LH release patterns for the two groups were similar. The response was quite different in the OVX non-SP mice as the first hour of release was only half that found in the OVX-SP. In the OVX non-SP group, the second episodic release of LH also quickly plateaued and remained elevated after the stimulus was removed. This peculiar pattern of LH release likely

reflects the lack of E2 modulation that was afforded the proestrous and OVX-SP mice. Because the responses in the proestrous and OVX-SP groups were similar in pattern (although not in magnitude) the OVX-SP protocol was adopted for studying LH secretion in aged mice in study No. 3.

Similar to the results of study No. 1, AP from aged mice, especially those subjected to long- and medium-term E2 exposure showed impaired LH secretory ability during the protein-dependent part of the release. In general, the AP from the aged mice exhibited significant releases in the first hour of stimulation, except for the aged group that was OVXed 2 months before sacrifice. These results confirm that exposure of the H-P axis to E2 does significantly alter LH function. Futhermore, the adverse influence of E2 appears to occur before middle age [5,9]. This was evident when AP from aged (18-month-old) mice OVXed at 8 months of age and given oil replacement, responded less to LHRH stimulation than AP from comparably treated mice OVXed at 3 months of age. The early influence of E2 on LH function was also apparent when no differences in LH release were found in the two aged groups OVXed at 8 months, despite one group having received E2 replacement for 8 months and the other only oil. The degree of impairment here was not as great, however, as that found in the aged group OVXed at 16 months or those OVXed at 3 months and given E2 until 16 months of age. Similarly, AP from aged mice OVXed at 16 months released significantly less LH than the aged group that were OVXed at 3 months and given long-term E2 replacement.

These results also suggest that other factors play a role in neuroendocrine aging that were not mimicked by weekly E2 injections. The amount of E2 that we injected (1 μg) was sufficient to induce LH surges in OVXed mice primed with E2 implants [27]. In our study, the injection produced plasma E2 levels in the high proestrous range (64 $\pm$ 13 pg/ml) for only the day of injection, but the low circulating levels of E2 found during the remainder of the estrous cycle were not duplicated by the single E2 injections. The longevity of the estrous cycles (4—5 days) in young mice may be of greater importance since OVXed mice injected with E2 on a weekly basis would experience fewer cyclic elevations in E2. Conversely, by 1 year of age estrous cycles in the mouse become erratic and lengthen. Therefore, regular weekly E2 injections probably subject older OVXed mice to more episodes of elevated E2 levels than usual. Whether the neuroendocrine axis in aged mice is more susceptible to the cyclic presence of E2 is not known, but the long-term elevation in plasma E2 experienced by aged mice either in constant estrous, or fed high levels of E2, significantly reduces the total number of estrous cycles supported in these mice upon receipt of young donor ovaries [24].

It can be argued that AP subjected to a weekly bolus of E2 alone would exhibit greater neuroendocrine impairment than intact cycling mice due to the omission of progesterone from the weekly steroid injection. Progesterone, in sufficient quantities and ratio to circulating E2 levels, is antagonistic to the harmful effects of E2 on the neuroendocrine axis [24,29]. The absence of progesterone did not appear to be a

factor here, however, since AP from aged mice OVXed at 16 months responded less to LHRH than AP from aged mice OVXed at 3 months and given weekly E2 replacement. It was also apparent that, by 18 months of age, LH secretory function in long-term OVXed mice had also been affected by non-ovarian influences. As expected, AP from aged mice OVXed at 3 months and given weekly E2 injections responded poorer to LHRH than AP from identically treated mice that received only the vehicle. The magnitude of the dual response from the latter group, however, did not match that of the AP from young OVX-SP mice. In fact, the response resembled that of proestrous animals in study No. 2, in both magnitude and pattern. A number of factors could contribute to non-ovarian related aging of the AP such as fatigue of the secretory machinery involved in the long-term, post-castration secretion of LH. Diet also affects aging, therefore, the increased caloric intake from long-term oil injections could also contribute to the aging process of the AP independent of E2 influence.

In summary, these studies demonstrated that the perifusion technique can be used to demonstrate age-related changes in LH secretory function. It is apparent that E2 is a prominent factor in the aging process of the AP, because altered LH secretion consistently occurred during the protein-synthesis dependent phase of LH secretion. Numerous questions can now be asked about the biosynthetic process(es) involved. The synthesis of replacement LH does not appear to be a limiting factor since the AP of aged mice contain more LH than young AP. The mechanism(s) involving LHRH receptor dynamics, the transformation of stored LH to a releasable form, and the transport of LH secretory granules to the cell membrane require further examination.

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