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The Increase of Anterior Pituitary Dopamine in Aging C57BL/6J Female Mice Is Caused by Ovarian Steroids, Not Intrinsic Pituitary Aging

Nancy Telford[a], *Charles V. Mobbs*[a], *Yagya N. Sinha*[b], *Caleb E. Finch*[a]

[a] Department of Biological Sciences and the Andrus Gerontology Center, University of Southern California, Los Angeles, Calif.; [b] Whittier Institute for Diabetes and Endocrinology, Scripps Memorial Hospital, La Jolla, Calif., USA

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Abstract. We describe how the increase of anterior pituitary dopamine (DA) during aging in female mice is related to altered secretion of ovarian steroids during reproductive senescence. A number of age-correlated neuroendocrine changes in female rodents result from cumulative exposure to ovarian steroids over a lifetime of estrous cycles, or from the altered pattern of ovarian steroid secretion concomitant with reproductive senescence. Pituitary DA has been shown to increase with age in female rats. To examine how the age-correlated increase of pituitary DA may depend on estradiol (E2), we measured pituitary DA and serum prolactin (PRL) in the following groups of female mice: young (7 months) cycling, middle-aged (14 months) cycling and non-cycling, old (17 months) non-cycling, old (17 months) ovariectomized (OVX) at 4 months, and young mice given 0.2 mg E2 valerate or E2 implants. Mice from some of these groups were OVX 1, 4 or 8 weeks before sacrifice. Compared with young controls, 14-month-old cycling or non-cycling mice had 3-fold higher pituitary DA, and 17-month-old non-cycling mice had 5-fold higher pituitary DA. OVX for 2 or 13 months before sacrifice abolished the effect of age: OVX of young mice had no effect on pituitary DA. Three weeks after implantation of E2 into OVX young mice or 7 weeks after injection of E2 valerate in intact young mice, pituitary DA was elevated. The E2-sensitive fraction of pituitary DA does not appear to decrease PRL secretion. We conclude that the age-correlated increase in pituitary DA is primarily dependent upon the effectively enhanced estrogenic stimulation concomitant with reproductive senescence, rather than upon intrinsic pituitary aging or irreversible effects from exposure to E2 over a lifetime of estrous cycles.

Dopamine (DA) levels in the anterior pituitary increase 75–300% during aging in male [37] and female [2, 9] rodents. This increase, the largest reported neurochemical change of aging [42], is puzzling because DA is strongly implicated as an inhibitor of prolactin (PRL) secretion by the pituitary, yet circulating PRL and pituitary DA both increase with age in rats [9, 26]. Moreover, the age-related increase of pituitary DA is in an unexpected opposite direction to the widely observed effects of age in reducing hypothalamic DA and DA in the blood of the hypothalamic-hypophyseal portal system, the only known source of pituitary DA [24, 41].

Interactions between hypothalamic DA and PRL are altered in several ways during aging. The decreased DA in the portal blood is a likely factor in the age-related elevations of circulating PRL seen in rats, since DA inhibits the secretion of PRL by the pituitary in many assay systems [19, 27, 40]. The well-established inverse relationships between portal blood DA and PRL secretion thus make the age-related increase in pituitary DA seem paradoxic. However, pituitary DA levels altered by pharmacologic means can vary widely without relation to circulating PRL [10]. Aging male mice have 75% increases of pituitary DA [37] without changes in plasma PRL [15]; more striking examples are seen in female mice (see Results).

Ovarian steroids also influence the regulation of PRL secretion by DA. Previous studies show that treatment of rodents with moderate doses of estradiol (E2) for 1–3 weeks increases PRL secretion [6, 28], and that prolonged treatment will induce PRL-secreting pituitary tumors (lactotroph adenomas) [12, 20, 50]. At the hypothalamic level, changes in DA are inverse to circulating PRL. In young rats, 2 μg E2/day for 14 days reduced DA in the median

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eminence [11], whereas ovariectomy (OVX) for 3 weeks increased DA at this site [51]. Pharmacologic studies also show effects of E2 on PRL regulation. Chronic E2 prevents nomifensine, an agent which stimulates the release of DA from the median eminence, from decreasing PRL secretion; however, it did not alter the ability of bromocriptine, a DA agonist, to inhibit PRL secretion, suggesting that E2 treatment alters the ability of the median eminence to secrete DA [5]. Reports on the response of portal blood DA to E2 treatments are inconsistent, and include no change [39], decreased [8] or increased [23] DA levels. Because chronic elevations of E2 can prematurely induce many neuroendocrine changes characteristic of female rodent aging [14], elevations of pituitary DA similar to those seen with age might be expected in response to sustained E2. Consistent with this view, aging acyclic rats exposed to sustained endogenous E2 from polyfollicular ovaries had 3-fold higher pituitary DA than cycling rats of the same age [9]. However, daily treatment of young rats with 25 μg E2 benzoate/kg or 10 μg E2 for 5–7 days appeared to decrease pituitary DA [7, 23].

To further resolve the relationships between aging, cycling status, estrogenic exposure and pituitary DA, we have studied C57BL/6J mice, an inbred strain with a 30-month average lifespan [14, 33]. These mice achieve fertility and maximum regularity of 4–5-day estrous cycles by 4–7 months [44]. Subsequently, between 8 and 12 months, estrous cycles lengthen. By 15 months, most mice have ceased cycling [30, 33] and enter a state of persistent vaginal cornification (PVC), characterized by moderate, sustained levels of plasma E2 secreted by the polyfollicular but anovulatory ovaries. Plasma progesterone, LH and PRL remain low during PVC in mice [16, 17]. Until 18 months (the age studied here) few mice have pituitary tumors or elevated PRL [16, 34]. Generally, PVC has ended by 24 months, when pituitary tumors (lactotroph adenomas) occur in >50% of female mice of this strain [44]. These tumors and many other neuroendocrine age changes of females are prevented if mice are OVX when young [14, 30]. Because chronic OVX can retard aspects of neuroendocrine aging, and because chronic elevations of E2 can prematurely induce many of these changes in young rodents, long-term exposure to ovarian E2 may be a major factor in neuroendocrine aging of female rodents [14]. This suggests that the age-correlated increase in pituitary DA in female rodents may also be due to long-term exposure to ovarian E2, rather than intrinsic pituitary aging or short-term exposure to ovarian E2.

The following studies examined the effects of E2 treatment and acute and chronic OVX on pituitary DA and serum PRL levels in young and aging mice. We report that the age-correlated increase in pituitary DA appears to be due primarily to altered ovarian secretion during aging, rather than intrinsic pituitary aging or long-term exposure to ovarian E2.

Materials and Methods

Animals and Treatment

Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, Me., USA) were acclimated to colony conditions (12 h light, 12 h dark, lights on at 06.00 h; Purina lab chow and water ad libitum) for at least 1 month before study. All mice were singly housed.

The ovarian status of middle-aged mice (retired breeders; 14 months at sacrifice) was classified by daily vaginal smears to obtain groups that were cycling (>3 consecutive, 5-day cycles) or acyclic (PVC, ≥3 weeks of cornified vaginal smears). Young (6–7 months) cycling mice were studied without reference to the day of the estrous cycle, as pilot studies showed no effect of day of the estrous cycle on pituitary DA.

Two groups of mice, young (7 months, virgin, showing regular estrous cycles) and aging (17 months, virgin, acyclic, PVC; ages given are at time of sacrifice) were ovariectomized (OVX) 1, 4, or 8 weeks before sacrifice, or OVX 1 week and given three 18-mm Silastic E2 implants for 3 weeks before sacrifice. These implants result in modestly elevated plasma E2 similar to levels found in aging PVC mice [28]. A third group, long-term OVX (17-month-old mice, OVX at 4 months) was given three 18-mm E2-containing Silastic implants (ca. 20–25 pg E2/ml plasma) [18] for 3 weeks. These implants were removed 1, 4 or 8 weeks before sacrifice. Another young group was given a subcutaneous depot injection of 0.2 mg E2 valerate in 0.1 ml peanut oil either 7 weeks before sacrifice, or were injected, OVX 7 weeks later and sacrificed 4 weeks after OVX, i.e. 11 weeks after injection of E2 valerate. This treatment of E2 valerate in intact mice results in a long-lasting (≥6 months) polyfollicular, acyclic state similar to the state of PVC found in aging mice [31].

Mice were killed by decapitation. Trunk blood was collected and the serum separated and stored at –70 °C until assayed for LH and PRL. The anterior pituitary was separated from the neurohypophysis; the arcuate-median eminence was dissected with fine surgical scissors. Tissues were rapidly frozen and stored at –70 °C. Mice with gross pathologic lesions were culled at necropsy [35].

Tissue Preparation

Tissues were homogenized in 100 μl 0.1 *N* HCl, 1 m*M* EDTA, 0.1 m*M* ascorbic acid containing 4 ng/ml 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard [38]. The samples were homogenized in 1.5-ml conical plastic tubes (Sarstedt, FRG) using a closely fitting, motor-driven teflon pestle. Each sample was homogenized with 10 strokes, frozen on dry ice, thawed and re-homogenized with 5 strokes. Two 5-μl aliquots of the homogenate were saved for protein determination [3] and the remainder was centrifuged at 35 *g* for 5 min. The supernatants were frozen at –70 °C overnight, then extracted for catechols (see below). DNA was measured in the pellet [4].

Chemicals and Sources

(–)-Arterenol bitartrate hydrate (norepinephrine, NE) and 3-hydroxytyramine-HCl (DA) were obtained from Calbiochem-Behring Corp., La Jolla, Calif., USA; DHBA from Sigma Chemical Co., St. Louis, Mo., USA; octyl sodium sulfate from Eastman Kodak Co., Rochester, N.Y., USA; acid-washed alumina from Bioanalytical Systems, Inc., West Lafayette, Ind., USA. Other chemicals were of the highest grade available. All weights given are for the

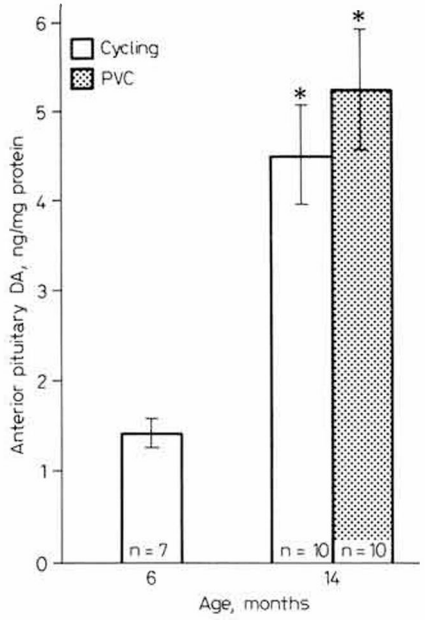

Fig. 1. The effects of age and cycling status on anterior pituitary DA. Means ± SEM. *$p < 0.01$ for effect of age, no effect of cycling status.

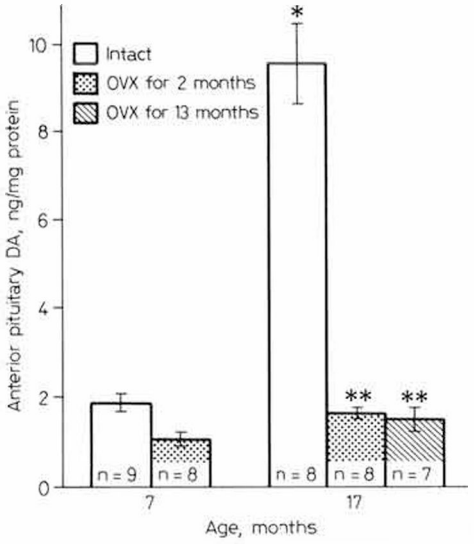

Fig. 2. The effects of age and OVX for 2 or 13 months on anterior pituitary DA. Means ± SEM. *$p < 0.05$ versus 7-month-old intact mice; **$p < 0.05$ versus intact mice of the same age (Student-Newman-Keuls test).

free compound. Water used for the mobile phases was deionized then distilled twice over $KMnO_4$ to remove impurities which may increase the baseline noise of the electrochemical detector.

Extraction of Catechols

NE and DA were extracted by binding to alumina using a modification of the method of *Felice* et al. [13] and *Osterburg* et al. [38]. Unless otherwise noted, all steps were done at room temperature and all centrifugations were done in a tabletop centrifuge. 1.0 ml of 0.5 *M* Tris (pH 8.6) was added to each sample, followed by 25 ± 5 mg alumina. The tubes were tightly capped and shaken vigorously in a mechanical shaker for 10 min. After 1 min centrifugation, the buffer was aspirated from the alumina and discarded. The alumina was washed twice with 1.0 ml H_2O, then transferred quantitatively via 3 × 0.3 ml H_2O to an RC-58 filter apparatus (Bioanalytical Systems). The filter apparatus was centrifuged 5 min to rid the alumina of H_2O. Catecholamines were eluted from the alumina by the addition of 135 µl 0.1 *N* HCl containing 10 m*M* sodium metabisulfite and 2 m*M* EDTA. The eluates are stable for ≥ 3 days on ice if protected from light.

Chromatography

100 µl of each sample was injected into a high-pressure liquid chromotograph (Altex, Berkeley, Calif., USA) equipped with a 5-µm C-18 reversed-phase precolumn and column (Brownlee Labs, Inc., Santa Clara, Calif., USA) and an electrochemical detector with a glassy carbon electrode (Bioanalytical Systems). The mobile phase was 50 m*M* potassium phosphate, 2 m*M* EDTA, 40 mg/l octyl sodium sulfate, pH 3.0, to which methanol was added to a final concentration of 12%. Flow rate was 1.5 ml/min and detector potential was +0.70 V. For the quantification of compounds in tissues, the ratio of sample peak height to the peak height of the internal standard was calculated, and this ratio was compared to a standard curve generated from the ratio of the peak height for known amounts of NE and DA to internal standard peak height [38].

Hormone Assays

PRL was assayed in a single run as described in [47]. Intra-assay coefficients of variation were 11% for samples greater than 4.0 ng/ml and 20% for samples less than 4.0 ng/ml. The detection limit was less than 4.0 ng/ml. LH was assayed in a single run as described in [28]. Intra-assay coefficients of variation were 4.7% for samples over 40 ng/ml and 27% for samples under 40 ng/ml. The detection limit was 10 ng/ml.

Statistics

The Statistical Analysis System [22] was used for analyses of main effects and interactions (General Linear Model, GLM), followed by Student-Newman-Keuls mutiple comparison tests when appropriate.

Results

Effects of Age and Cycling Status

By 14 months of age, anterior pituitary DA content increased 3-fold above 6-month-old cycling mice (fig. 1). This age-related increase was similar in aging mice which still had regular 5-day cycles or which had recently ceased cycling and were in the initial stages of PVC (see Introduction). The pituitary DA of young cycling mice did not differ between estrus and diestrus (data not shown).

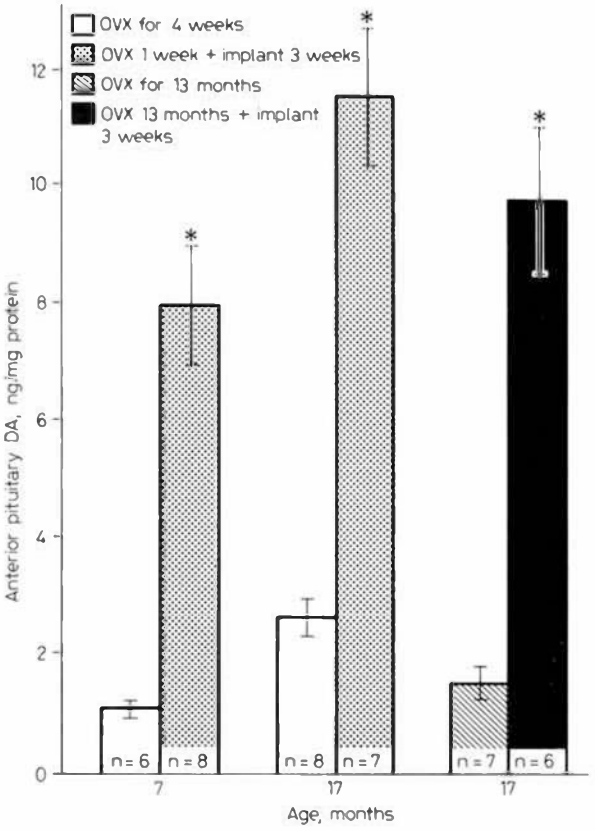

Fig. 3. The effect of E2 implants on anterior pituitary DA content. One week after OVX, mice each received three 18-mm E2-containing silastic capsules that yield 20–25 pg E2/ml serum [18], and were sacrificed 3 weeks later. Control mice were OVX 4 weeks before sacrifice. Means ± SEM. *$p < 0.001$ for main effect of implants.

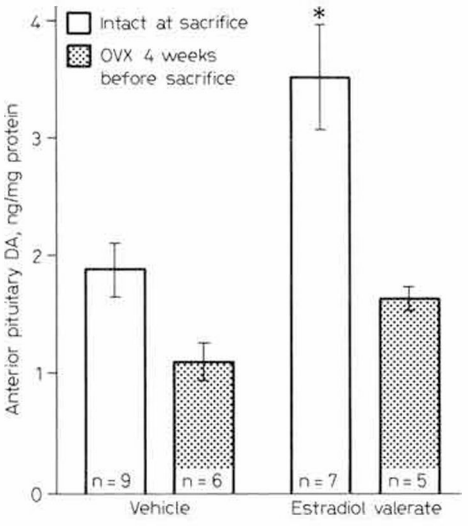

Fig. 4. The effect of E2-valerate injections and OVX on anterior pituitary DA in 7-month-old mice. Mice were injected with 0.2 mg E2-valerate or oil vehicle. Seven weeks after the injection, mice were either sacrificed, or OVX and sacrificed 4 weeks after OVX (11 weeks after injection). Means ± SEM. *$p < 0.05$ versus intact oil-injected group (Student-Newman-Keuls test).

Effects of Short- and Long-Term OVX

The age-related increase of pituitary DA was prevented by long-term (13 months) or acute (2 months) OVX of 17-month-old mice (fig. 2). This result suggests that the presence of the ovary rather than age per se was responsible for the increased pituitary DA. OVX of young mice for 2 months did not significantly alter pituitary DA levels (fig. 2).

E2 Treatment of Young Mice

We then investigated if treatment of young mice with E2 could elevate pituitary DA to levels observed in aging mice. Two routes of E2 administration were used. Implants of E2 given to young OVX mice for 3 weeks increased pituitary DA 6-fold (fig. 3). Alternatively, intact mice were given subcutaneous depot injections of E2 valerate; pituitary DA increased 2-fold when measured 7 weeks later (fig. 4).

E2 valerate injections into intact mice produce alterations of neuroendocrine functions that persist long (at least 6 months) after the exogenous E2 has cleared, including the induction of a permanent PVC-like syndrome accompanied by moderate, tonic elevations of plasma E2 from the polyfollicular ovaries [29]. To determine if the effects of E2 valerate were direct or indirect due to the presence of polyfollicular ovaries, some of the E2-valerate-injected mice were OVX 7 weeks after injection and killed 4 weeks later. After OVX, pituitary DA subsided to levels of untreated intact young mice (fig. 4). Thus, the elevation in pituitary DA induced in young mice by E2-valerate requires the continued presence of E2 from the polyfollicular ovaries. A few E2-valerate-injected mice (3 of 20) had enlarged pituitaries or possible tumors and were excluded from analysis. There were no differences between controls and treatment groups in protein content or protein: DNA ratio of pituitary homogenates (data not shown).

Time Course after OVX

The time course of changes in pituitary DA after OVX was examined in 7- and 17-month-old mice to evaluate possible influences of age on the stability of the E2-sensitive fraction of pituitary DA (fig. 5). In young mice, pituitary DA did not change from levels in intact mice for up to 8 weeks after OVX, and was indistinguishable from levels seen in unimplanted long-term OVX mice that were OVX as young mice 56 weeks (13 months) before. Following OVX, the elevated levels of pituitary DA in aging mice decreased 30% at the end of 1 week and 75% by 4 weeks. There

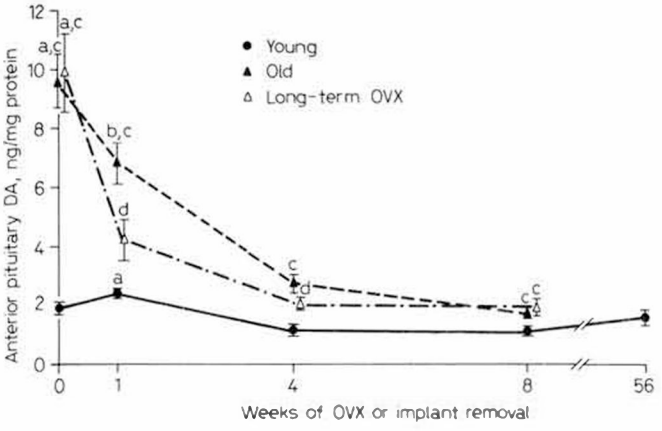

Fig. 5. Time course of the response of anterior pituitary DA levels to OVX of 7 or 17-month-old intact mice or the removal of E2-containing implants from 17-month-old long-term OVX mice. Unimplanted long-term OVX mice are designated as 56-week OVX young mice. Vertical lines indicate SEM. [a] $p < 0.05$ versus 8-week time point of the same group; [b] $p < 0.05$ versus 0 and 8-week time point of the same group; [c] $p < 0.05$ versus 7-month-old mice at the same time; [d] $p < 0.05$ versus 7 and 17-month-old mice at the same time (Student-Newman-Keuls test). n = 6–9 per cell.

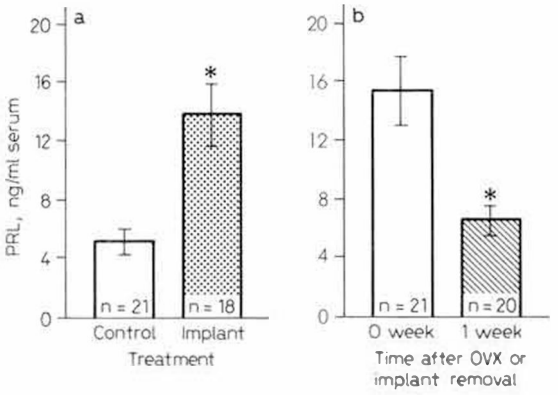

Fig. 6. a Effect of E2-containing implants on serum PRL. Implanted mice received three 18-mm E2-containing silastic implants 1 week after OVX and were sacrificed 3 weeks later. Control mice were OVX 4 weeks before sacrifice. Because age had no effect on serum PRL, all ages were combined. Means ± SEM. *$p < 0.001$ for effect of implants. **b** Effect of removal of ovaries or implants on serum PRL. Because age had no effect on PRL, all ages were combined. There was no further decrease in serum PRL after 1 week of OVX or implant removal. Means ± SEM. *$p < 0.001$ for effect of removal of the estrogenic stimulus.

was no further change through 8 weeks after OVX. The initial DA decrease approximated exponential decay with a half-life of 10.0 days ($r = -0.54$).

When 17-month-old long-term OVX mice were given E2-containing implants for 3 weeks, pituitary DA increased to levels seen in 17-month-old intact mice (fig. 5). When the E2 implants were removed, pituitary DA declined gradually, reaching basal levels by 8 weeks. The decline was more rapid in long-term OVX mice than in intact aging mice, with a half-life of 3.1 days [$p < 0.001$ for interactive effect of time after OVX or implant removal and previous ovarian state (intact or long-term OVX) on pituitary DA]. By 8 weeks after implant removal, pituitary DA was indistinguishable from the levels in 8-week OVX aging mice.

Hypothalamic Catecholamines

The DA and NE levels in the arcuate-median eminence were 33.5 ± 7.4 ng/mg protein (n = 9) and 20.1 ± 1.0 ng/mg protein (n = 8), respectively, in young intact mice. They were unchanged by age, E2 treatment, OVX or implant removal, unlike anterior pituitary DA.

Serum PRL and LH

Serum PRL did not change with age in intact mice (data not shown), as observed previously [16], but was increased 2-fold by E2 implants and was decreased by OVX or implant removal (fig. 6). There was no inverse correlation between serum PRL levels and pituitary DA content within any of the individual groups, or when all groups were combined. This result diminishes the possibility that most of the DA in the anterior pituitary influences PRL secretion (see Discussion).

Following OVX, serum LH levels increased in all groups, as expected (data not shown). In young mice, LH was elevated above the levels in intact animals by 4 weeks after OVX, while in aging mice, LH increased more slowly; increases were significant only by 8 weeks following OVX of 17 month-old intact mice or removal of E2 implants from long-term OVX aging mice (data not shown). These results agree with previous studies [17, 45].

Discussion

The age-related increase of anterior pituitary DA in female rats [2, 9] is extended here to females of another species, the mouse. The present study suggests that the age-correlated increase in pituitary DA can be explained largely by the altered patterns of ovarian steroid secretion characteristic of reproductive senescence in female rodents. As cycles grow longer and cease during reproductive senescence, the ovaries secrete less progesterone, while E2 levels stay moderately elevated; thus target tissues may experience a relatively unopposed and effectively enhanced estrogenic

stimulation [32]. It is this persistent, effectively enhanced estrogenic stimulation which appears to be largely responsible for increased pituitary DA, rather than an intrinsic pituitary age change or the cumulative exposure to E2.

Three lines of evidence support this conclusion. First, OVX greatly reduces the effect of age on pituitary DA, although, in some cases (fig. 5), a small difference remains. If the increase was due to intrinsic aging of the pituitary or the cumulative exposure to E2, removal of the ovaries of aging mice should have little effect. Second, simulating the moderately elevated E2 levels produced by polyfollicular ovaries by the use of E2 implants led to increases of pituitary DA in young mice similar to increases in aging mice. These implants produce serum E2 levels comparable with those seen in intact mice on proestrus [18]. The cumulative E2 levels from 21 days of these implants is certainly less than the lifetime exposure during 50 estrous cycles [14]. Finally, the single injection of E2 valerate also produces a polyfollicular acyclic state similar to the PVC state of aging mice [29]. This treatment also elevates pituitary DA. It should be noted, however, that elevating E2 did not elevate pituitary DA quite as much in young mice as in aging mice (fig. 3), nor did OVX of aging mice completely reduce pituitary DA levels to those measured in young mice (fig. 5). Therefore, intrinsic pituitary aging and/or the cumulative exposure to E2 may also contribute to age-correlated increases in pituitary DA, though such contributions appear to be minor compared to the effect of enhanced estrogenic stimulation.

Similar elevations of pituitary DA occured in both cycling and non-cycling (PVC) groups of 14-month-old mice (fig. 1). Because E2 implants, which produce sustained elevations of E2 similar to those seen in PVC, caused pituitary DA elevations in young mice (fig. 3), we expected that *cycling* mice would have lower pituitary DA than PVC mice of the same age. This result merits further study, but suggests that even regularly cycling ovaries in 14-month-old mice may secrete effectively enhanced levels of E2 which induce age-correlated biochemical changes [32].

A difference was found in the dynamics of pituitary DA between intact 17-month-old and E2-implanted, long-term OVX 17-month-old mice. While both 17-month-old intact mice and implanted long-term OVX mice had similar levels of pituitary DA before and 8 weeks after removal of the ovaries or implants, the initial decline in pituitary DA was more than twice as rapid in long-term OVX mice following implant removal (half-life = 3.1 days) than in the aging mice following OVX (half-life = 10.0 days). This difference in the rate of decrease of pituitary DA is unlikely to result from effects of long-term OVX on the clearance of plasma E2, since the half-life of serum E2 after OVX is less than 1 h [25]. We speculate that the slower efflux of DA from the pituitaries of aging intact mice is the result of an alteration in cellular characteristics, such as an increasing number of lactotrophs. Old rats have an increased number of lactotrophs, an age-related change which is attenuated by long-term OVX [48].

The increase in pituitary DA in response to E2 treatment could result from increased secretion of DA from the hypothalamus and/or increased uptake and storage of DA by pituitary cells; the anterior pituitary may be incapable of DA synthesis from tyrosine as it appears to lack tyrosine hydroxylase [43]. The gradual depletion of the E2-sensitive fraction following the removal of E2 is more likely to be related to a property such as pituitary DA storage capacity, rather than to clearance of plasmsa E2 or DA turnover, since the decrease in pituitary DA following E2 removal occurs 2–3 orders of magnitude more slowly than the clearance of plasma E2 (see above) or the turnover of DA [1, 37].

Unlike DA in the pituitary, levels of DA and NE in the arcuate-median eminence were unaffected by aging, E2 treatment and OVX; thus, the increase of pituitary DA with age in female mice can occur in the absence of gross disturbances in hypothalamic DA levels. The absence of age changes in median eminence catecholamines of middle-aged female C57BL/6J mice contrasts with previous studies in middle-aged and older rats in which 20–30% decreases are commonly reported [46, 52]. In male C57BL/6J mice, hypothalamic catecholamine metabolism does not decrease until after 24 months [37], an older age group than that studied here.

Anterior pituitary DA appears to exist in two fractions. The smaller, basal fraction is found in young cycling and OVX mice, and is revealed in aging mice following 8 weeks or more of OVX. The E2-sensitive fraction, which is potentially much larger, is found in aging intact mice as well as in young and long-term OVX mice treated with E2. Since prolactinemia occurs concomitantly with elevated pituitary DA in aging rats [2, 9] and since we found no inverse relationship between pituitary DA and serum PRL in mice, we conclude that the larger, E2-sensitive fraction of pituitary DA does not suppress PRL secretion, while the smaller, basal fraction may.

The function and location of these two pituitary DA fractions remain unknown. It is tempting to conclude that pituitary DA occurs mainly in the lactotrophs because of their higher content of DA receptors [21], and because most pituitary DA co-sedimented with a heavy-particle fraction containing PRL, but not other pituitary hormones, during centrifugation of pituitary homogenates from young rats on sucrose gradients [31]. In aging rats, about half of the DA sedimented more slowly, not in association with PRL [2]. It remains to be established which pituitary cell types contain these two density-defined subcellular DA compartments, as well as the two DA fractions described here. Finally, we note that GABA, another factor secreted by the hypothalamus into portal blood, also can inhibit PRL secretion and, moreover, is increased 6-fold in the pituitary of male rats by

7 days of E2 treatment [36]. If the additional pituitary GABA and DA accumulate in the loci of lactotrophs where these compounds would normally inhibit PRL secretion, the E2 must decouple the inhibitory mechanism, since serum PRL also increases with E2 treatment.

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Nancy Telford,
Andrus Gerontology Center,
University of Southern California,
Los Angeles, CA 90089-0191 (USA)