# Luteinizing Hormone Response to N-Methyl-D, L-Aspartic Acid in the Presence of Physiological Estradiol Concentrations: Influence of Age and the Ovary (43898)

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*Abstract.* **We have previously reported that the pituitary of intra-atrially cannulated old female C57BL/6J mice is as capable of responding to a GnRH challenge as is that of young females (10). We have observed elevated luteinizing hormone (LH) levels in ovariectomized (OVX) intra-atrially cannulated mice. Sustained physiologic levels of estradiol ($E_2$) for 6 days suppressed circulating LH to intact levels. However, in that model, a bolus of $E_2$ following $E_2$ priming was unable to elicit an LH surge (Joshi *et al.*, unpublished findings). The present studies were designed to examine: first, whether GnRH neurons are competent to release GnRH in the presence of tonic physiologic levels of $E_2$ and, second, whether either age or the ovary can influence GnRH neuronal responsiveness. The N-methyl-D, L-aspartic acid (NMA)-evoked GnRH response was assessed indirectly by measuring LH in two groups of OVX C57BL/6J mice: short-term OVX (S-OVX) (1 week) mice were either prepubertal (5 weeks), postpubertal (10 weeks), young (5 months), middle aged (12 months), or old (24 months). Long-term OVX (L-OVX) mice were either young (5 months), or old (24 months) and OVX at puberty; middle-aged L-OVX mice were OVX at 8 months and examined at 12 months of age. Animals were administered physiologic levels of $E_2$ by subcutaneous silastic capsule for 1 week before testing. LH secretion was inhibited by $E_2$ in S-OVX mice of all ages. In no case did NMA overcome this inhibition in $E_2$ primed S-OVX females. $E_2$ also inhibited LH secretion in L-OVX mice of all ages, but NMA was able to overcome the $E_2$ inhibition of LH secretion in L-OVX mice (young: 0.5 ± 0.1, 0.84 ± 0.19 ng/ml, first and second challenge, respectively; middle-aged: 0.46 ± 0.1, 1.08 ± 0.16 ng/ml; and old: 1.44 ± 0.19, 0.99 ± 0.27 ng/ml). This last effect was independent of animal maturity at the time of OVX or animal age at the time of experiment. These findings suggest that although the ovaries in the 24-month-old S-OVX mice had not produced enough $E_2$ to alter the vaginal cytology for 2 ± 0.5 months before the experiment, the ovarian modulation of the inhibitory effect of $E_2$ on NMA-induced LH secretion was still present. The nature of the ovarian factor(s) modulating this effect is unknown. These results demonstrate that in the intra-atrially cannulated female C57BL/6J mouse, the negative feedback effect of $E_2$ on hypothalamic GnRH release predominates and prevents the induction of an LH surge by a bolus of $E_2$. The ability of $E_2$ to inhibit the NMA response is mediated by the length of time between removal of the ovary and initiation of estrogen replacement, and this effect is independent of age.**

[P.S.E.B.M. 1995, Vol 209]

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Received July 14, 1994. [P.S.E.B.M. 1995, Vol 209]
Accepted January 30, 1995.

0037-9727/95/2093-0237$10.50/0

Age-related loss of reproductive function in female rodents is characterized by a decline in preovulatory luteinizing hormone (LH) secretion (for review see Ref. 1 and 2). A significant reduction in the LH surge in response to an estradiol ($E_2$) challenge has been demonstrated in rats (3–7). An attenuation of the $E_2$-induced LH surge has also been described in trunk blood samples in aged C57BL/6J mice (8, 9). In previous studies, we have found that in both young and old intra-atrially cannulated OVX, $E_2$-treated mice, the pituitary can respond to an exogenous challenge of GnRH with a LH response that is equal to that of the LH surge during a normal estrous cycle (10). Although pituitary function in our model is apparently not altered by either $E_2$ inhibition or age (10), the LH response to an $E_2$ bolus could not be elicited (Joshi *et al.*, unpublished findings). However, robust LH secretion was obtained in intra-atrially cannulated OVX young, middle-aged, and old animals in the absence of $E_2$ (this report). Thus, physiological levels of $E_2$ (i.e., those achieved with $E_2$ priming) are apparently mediating the inhibitory effect of factors associated with the cannulation procedure. $E_2$ has been shown to enhance the suppression of pulsatile LH release in several cases including the "stress" of fasting in rats (11), and chair restraint and insulin-induced hypoglycemia in monkeys (12, 13). The present studies were done in unrestrained, intra-atrially cannulated $E_2$-treated mice in order to examine whether the GnRH neurons are competent to release GnRH while being inhibited by tonic physiologic levels of $E_2$, and whether either age or ovarian status can affect this GnRH neuronal responsiveness.

N-methyl-D, L-aspartic acid (NMA) is a potent agonist of the neuroexcitatory amino acids aspartate and glutamate (14, 15). NMA can acutely stimulate luteinizing hormone (LH) secretion by acting at the hypothalamic level since it stimulates the release of gonadotropin hormone-releasing hormone (GnRH) *in vitro* (16–19). Its effect on LH release is blocked by a GnRH antagonist (20, 21). This action of NMA may be mediated by specific NMA receptors located on GnRH neurons or terminals, or associated afferents (22) in the medial basal hypothalamus and/or preoptic area (16–18, 23–25). NMA has been utilized as a neuroendocrine probe to assess the competency of GnRH neurons in low gonadotropin states such as in hypogonadal mice (22), prepubertal rats (21, 26–28), and peripubertal monkeys (20, 29–31).

In the present study, we have used systemic administration of NMA as a provocative test for GnRH neuron responsiveness. The GnRH response to NMA was monitored indirectly by measuring LH in serial blood samples obtained from C57BL/6J mice of varying ages. Animals were OVX either for 1 week (short-term OVX [S-OVX]) or for varying longer periods (long-term OVX [L-OVX]). Mice were administered tonic physiologic levels of $E_2$ for 1 week by implantation of an $E_2$ capsule either at the time of OVX of the S-OVX animals, or 1 week before testing of L-OVX mice. The results of these studies indicate that the $E_2$ inhibition of the GnRH response to NMA is modulated by the length of time the ovary has been absent and that this effect is independent of age.

## Materials and Methods

**Animals.** Virgin female C57BL/6J mice (Jackson laboratory, Bar Harbor, ME) were housed triply with bedding, food, and water (12:12-hr light:dark cycle) as described (32) in a limited access room restricted to mice used in this study. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals were used for animal husbandry and all protocols were approved by the McGill University and Royal Victoria Hospital Animal Care Utilization Committee. No parasitic, bacterial or viral pathogens were detected during the period of study in the colony as determined by a sentinel program. Three age groups representing different stages of reproductive aging were studied. Young mice (5 months) were regularly (4–5 day) cycling for at least 2 months as evaluated by daily vaginal smears; middle-aged mice (12 months) exhibited at least three long (5–7 day) or irregular cycles; and old mice (23–24 months) were acyclic and exhibited leukocytic vaginal smears for 2 ± 0.5 months before experimentation. Animals were cannulated intra-atrially 24 hr before sampling as previously described (10). Three different experimental paradigms were employed to assess the effect of age and the ovary on GnRH neuronal competence.

*Experiment 1: The effect of OVX on LH secretion without $E_2$ supplementation.* LH secretion was measured in young ($n$ = 3), middle-aged ($n$ = 3), and old ($n$ = 3) mice which were OVX for 1 week (S-OVX).

*Experiment 2: The effect of age on LH secretion and NMA evoked GnRH secretion with $E_2$ supplementation.* This was examined indirectly by measuring the LH response to saline or NMA in S-OVX young ($n$ = 8), middle-aged ($n$ = 8), and old ($n$ = 8) female mice. A group of prepubertal mice (5 week) ($n$ = 8) that had not started cycling until the time of the experiment and a group of postpubertal mice (10 week) ($n$ = 8) exhibiting a minimum of three consecutive 4- to 5-day cycles were also studied. Animals were implanted subcutaneously with a 10 mm-long silastic capsule (i.d. 0.04 in; o.d. 0.085 in, Dow Corning) containing 17β-estradiol which delivered tonic physiologic levels of $E_2$ for 1 week before testing as described previously (10).

*Experiment 3: The effect of the ovary on LH secretion and NMA evoked GnRH secretion with $E_2$*

*supplementation.* This was examined indirectly by measuring the LH response to saline or NMA in L-OVX (OVX at puberty, i.e., 7–8 weeks of age) young ($n$ = 8) and old ($n$ = 8) mice. Middle-aged mice ($n$ = 8) were OVX at 8 months in order to determine whether age at the time of OVX has an effect on the LH response. All animals were given tonic physiologic levels of $E_2$ for 1 week via silastic capsules before experimentation.

**Blood Sampling.** For Experiment 1, serial blood samples were obtained at 15-min intervals for 165 min. For Experiment 2 and 3, three serial baseline samples were taken at 15-min intervals. Either saline (4 ml/kg body wt) or NMA (20 mg/4 ml saline/kg body wt; Sigma Chemical Co., St. Louis, MO) were administered 10 min after the last baseline sample and either saline (4 ml/kg body wt) or NMA (40 mg/4 ml saline/kg body wt) were administered 80 min after the last baseline sample. Blood samples following administration of saline or NMA were taken 10 min later according to the protocol of Saitoh *et al.* (22). Remaining consecutive samples were taken at 20-min intervals. No overt behavioral changes due to administration of NMA were observed in any of the mice after either dose.

Samples were centrifuged and plasma stored at −70°C until assay. Blood cells were resuspended in an equal volume of charcoal stripped heparinized (2 U/ml) (33) human serum albumin (HSA) and returned to the host via the indwelling catheter. Sampling was always performed between 13:30 and 17:30 hr. At the end of the experiment, trunk blood was collected for serum $E_2$ measurement, and animals were necropsied according to our standard protocol (10).

**Radioimmunoassay.** Blood samples were measured in duplicate for LH with the kit for rat LH (NIDDK) with rat LH RP-2 standard. Sensitivity of the assay was 0.1 ng/ml. Values below the sensitivity of the assay were brought to the level of detectability of the assay (i.e., 0.1 ng/ml) for statistical comparisons. The intra- and interassay coefficients of variation were 5.03% and 7.8%, respectively. Serum concentration of $E_2$ was determined using 16α-iodo, 17β-[$^{125}$I]estradiol and an antibody to $E_2$ (Radioimmunoassay Systems Labs. Inc., Carson, CA). Sensitivity of the assay was 1.0 pg/ml $E_2$ and intra- and interassay coefficient of variation were 12.7% and 13.8%, respectively.

**Statistical Analysis.** Data are expressed as means ± SEM. A significant LH response was identified according to our criteria (10) of initial elevation greater than 20% of the baseline value. The amplitude of the LH response to the first NMA challenge was compared with the amplitude of the LH response to the second NMA challenge within each animal group by one-way analysis of variance (ANOVA). The amplitude of the LH response to either the first, or to the second NMA challenges, respectively, was compared among animal groups by one-way ANOVA according to the method of Saitoh *et al.* (22). A modified Bonferroni test was used for *post-hoc* comparisons using Instat Version 1.13 (GraphPad Software, San Diego, CA). Differences with $P$-values $<0.05$ were considered statistically significant.

## Results

**Experiment 1: The Effect of OVX on LH Secretion Without $E_2$ Supplementation.** An example of the LH secretion in young, middle-aged, and old S-OVX animals is shown in Figure 1. There was no significant difference between mean LH levels in young (0.49 ± 0.07 ng/ml), middle-aged (0.34 ± 0.08 ng/ml), and old (0.47 ± 0.15 ng/ml) animals. The maximum LH concentrations observed were also not significantly different among the three groups: 0.68 ± 0.4 ng/ml, 0.99 ± 0.2 ng/ml, and 0.89 ± 0.3 ng/ml, respectively. Thus, in the absence of the suppressive effect of $E_2$, OVX females of all age groups demonstrated similar LH responses.

**Experiment 2: The Effect of Age on LH Secretion and NMA Evoked GnRH Secretion with $E_2$ Supplementation.** Serum LH levels in S-OVX $E_2$-treated young, middle-aged, and old animals are shown in Fig. 2. LH levels in young (0.1 ± 0.01 ng/ml), middle-aged (0.1 ± 0.01 ng/ml), and old (0.14 ± 0.01 ng/ml) $E_2$ supplemented mice were suppressed compared with the S-OVX animals of same age group (compare with Fig. 1.). In no case was an LH response to NMA obtained in young, middle-aged, or old S-OVX animals (Fig. 2).

Serum LH levels in pre- and postpubertal S-OVX $E_2$-treated animals are shown in Figure 3. There was no significant difference between baseline LH levels in the prepubertal and postpubertal groups. In prepubertal mice a small, but significant, LH response to both challenges of NMA (0.18 ± 0.05 ng/ml, 0.25 ± 0.07 ng/ml, $P < 0.03$) was observed (Fig. 3), which contrasted with the lack of response to NMA in the postpubertal mice.

**Experiment 3: The Effect of the Ovary on LH Secretion and NMA Evoked GnRH Secretion with $E_2$ Supplementation.** Serum LH levels in L-OVX $E_2$-treated animals are shown in Figure 4. $E_2$ suppressed LH levels in young (0.1 ± 0.01 ng/ml), middle-aged (0.1 ± 0.01 ng/ml) as well as in old (0.2 ± 0.01 ng/ml) mice as effectively as in S-OVX animals of the same age groups (compare with Fig. 2). There was also no significant difference in the baseline LH levels between young and old animals.

The L-OVX group demonstrated a marked response to NMA compared with S-OVX animals of the same age groups (compare Fig. 2 and 4). There was a significant LH response to each of the two challenges

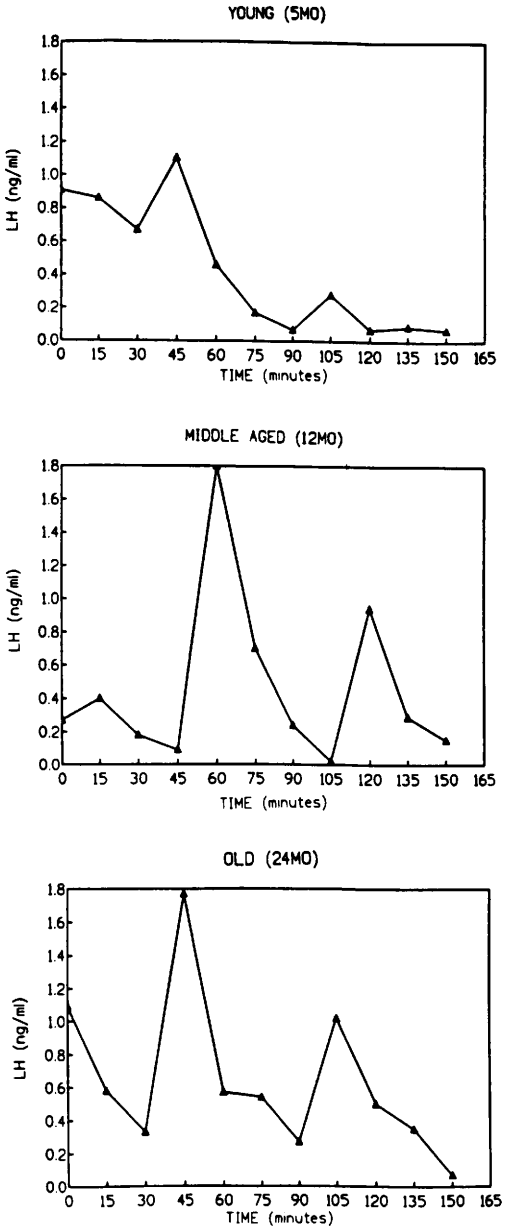


**Figure 1.** Representative profile of LH secretion in young, middle-aged, and old mice. Animals were OVX for 1 week (S-OVX) and did not receive exogenous $E_2$. Blood samples were obtained as described in the text.

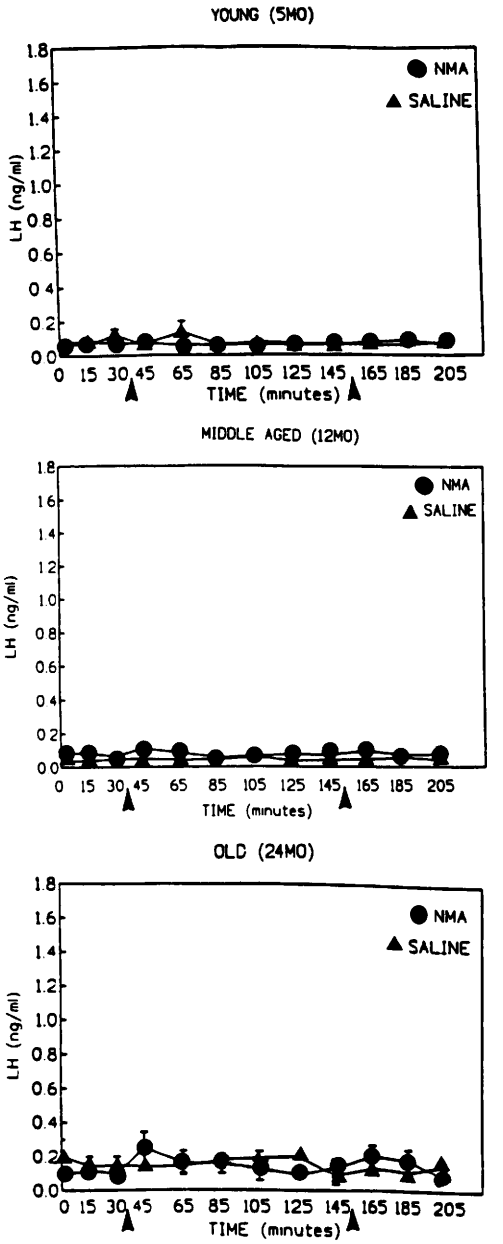


**Figure 2.** Effect of NMA on LH secretion in young, middle-aged, and old S-OVX mice. Animals were OVX and received $E_2$ for 6 days. Blood samples were obtained as described in the text. After three baseline samples, animals were challenged with NMA (20 mg/4 ml saline/kg body wt) or saline (4 ml/kg body wt) 10 min after the last baseline sample followed by a second challenge of NMA (40 mg/4 ml saline/kg body wt) or saline (4 ml/kg body wt) 80 min after the last baseline sample. Each data point is mean ± SEM of $n = 8$ for young, middle-aged, and old animals receiving NMA and $n = 4$ for the saline controls.

of NMA in both young (0.5 ± 0.1 ng/ml, 0.84 ± 0.19 ng/ml, $P < 0.03$) and old (1.44 ± 0.19 ng/ml, 0.99 ± 0.27 ng/ml, $P < 0.003$) L-OVX mice. The LH response to the first challenge of NMA was significantly greater in old mice than that of young animals ($P < 0.03$). No significant difference was observed in the LH response to the second challenge of NMA between the two age groups.

$E_2$ inhibited the secretion of LH in mice of all age groups. This effect was independent of the length of OVX before $E_2$ administration. However, there was a differential response to NMA which appeared to depend upon the time of OVX. Inhibition of NMA-stimulated LH secretion by $E_2$ was present in 10-week- 5-month- 12-month- and 24-month-old S-OVX animals. It was not present in 5- and 24-month-old mice if their ovaries were removed peripubertally (i.e., at 7–8 weeks of age). For this reason, we also tested whether the ovarian influence on $E_2$ inhibition of the NMA response is developmental. For this part of the study, animals that had regular ovarian function for at

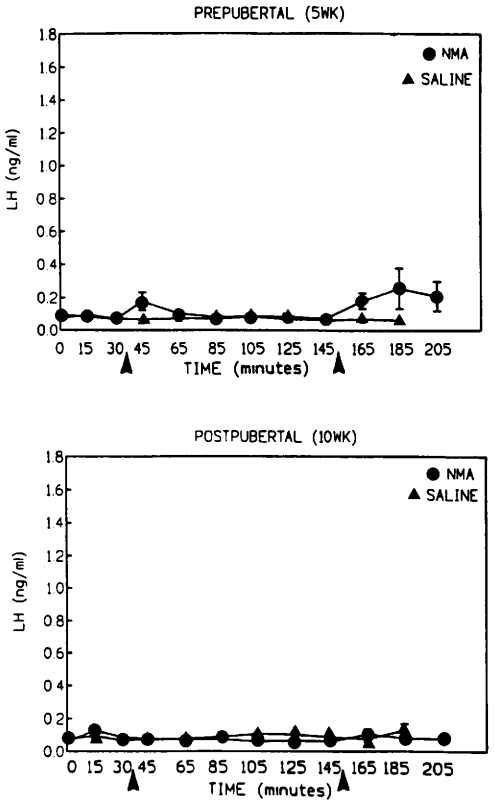


**Figure 3.** Effect of NMA on LH secretion in prepubertal and postpubertal S-OVX mice. See Figure 2 for details on NMA challenge. Each data point is mean ± SEM of $n = 8$ for both prepubertal and postpubertal animals receiving NMA and $n = 8$ for the saline controls.

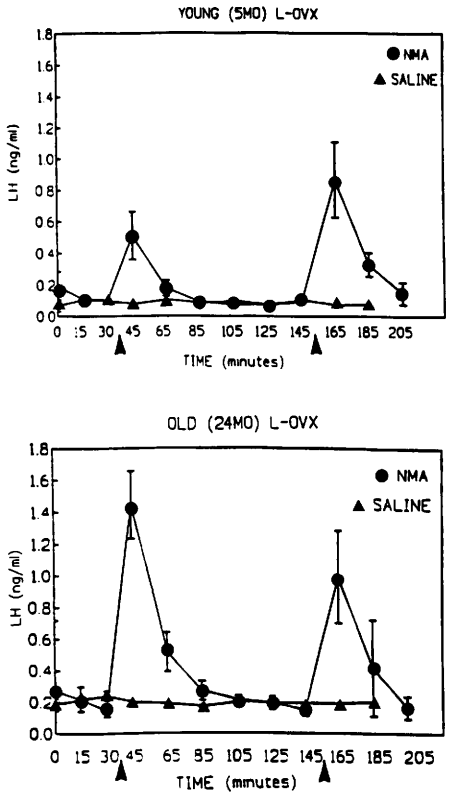


**Figure 4.** Effect of NMA on LH secretion in young and old mice OVX at puberty (L-OVX). Animals were OVX at 7–8 weeks of age and received $E_2$ for 6 days before the NMA test. Blood samples were obtained as described in text. Each data point is mean ± SEM of $n = 8$ for both young and old animals receiving NMA and $n = 4$ for the saline controls.

least 6 months before gonadectomy were OVX at 8 months of age, and the NMA response was tested at 12 months of age. A significant LH response was obtained in these middle-aged L-OVX animals to both challenges of NMA (0.46 ± 0.1 ng/ml, 1.08 ± 0.16 ng/ml) (Fig. 5). $E_2$ was *not* able to inhibit the NMA response in these L-OVX animals in contrast to the findings in S-OVX animals of the same age (compare with Fig. 2).

**Serum $E_2$ Levels.** The average serum $E_2$ levels achieved by the $E_2$ capsules were: 9.5 ± 4.5 pg/ml in prepubertal animals, 11.6 ± 3.3 pg/ml in postpubertal animals, 13.5 ± 2.9 pg/ml in young S-OVX and 21.5 ± 4.7 pg/ml in young L-OVX animals, 32.2 ± 8.1 pg/ml in middle-aged S-OVX and 18.6 ± 8.6 pg/ml in middle-aged animals OVX at 8 months, 23.5 ± 7.3 pg/ml in old S-OVX and 14.3 ± 4.5 pg/ml in old L-OVX mice. There was no significant difference in the serum $E_2$ levels among the different age/treatment groups ($P = 0.6$, ANOVA).

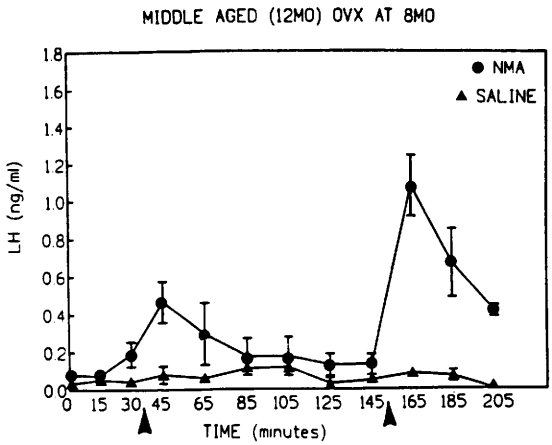


**Figure 5.** Effect of NMA on LH secretion in middle-aged mice OVX at 8 months and studied at 12 months of age. See Figure 2 for details. Each data point is mean ± SEM of $n = 8$ for animals receiving NMA and $n = 4$ for the saline controls.

## Discussion

This study demonstrates that, in intra-atrially cannulated mice, $E_2$ inhibition of basal GnRH secretion is not altered by age or the presence of the ovary. In S-OVX mice, $E_2$ inhibits the NMA stimulation of LH secretion. The response of GnRH neurons to NMA differs depending upon the length of time that the ovary has been absent. This study is the first to examine the effect of age on estrogen's negative feedback effect on LH secretion in the female C57BL/6J mouse.

In the absence of exogenous $E_2$ in the intra-atrially cannulated female C57BL/6J mouse, GnRH neurons

are capable of releasing GnRH regardless of age. Mean LH levels in old OVX mice without $E_2$ supplementation were similar in magnitude to young and middle-aged animals. This agrees with studies in noncannulated female CBA mice (34), and Long Evans rats (4, 35, 36). When physiological $E_2$ concentrations were maintained for 6 days, LH secretion was inhibited equally in all age groups and regardless of the length of OVX. Thus, there is apparently no significant effect of age or length of OVX on $E_2$ inhibition of LH secretion. This confirms previous reports in female mice (8) and rats (35–37). In addition, $E_2$ also inhibited the LH response to NMA in the S-OVX mice, regardless of age. These findings confirm the results of Saitoh *et al.* (22) demonstrating $E_2$ inhibition of NMA evoked LH release in young female C3H/HeH X 101H mice. The present studies extend these observations to middle aged and old animals. These data further demonstrate that L-OVX reverses the ability of $E_2$ to inhibit the NMA response.

Other studies have suggested that $E_2$ appears either to potentiate or to be necessary for NMA stimulation of LH secretion in short-term OVX rats (27, 38, 39). In those reports, the LH response was measured 2 days after priming with a single high dose of estradiol or estradiol benzoate. This regime may be more applicable to the study of the positive feedback effect of $E_2$. The study of Saitoh *et al.* (22) and the present study evaluate the negative feedback effect of $E_2$ on LH secretion; a much greater increase in serum LH after an NMA challenge was seen in OVX C3H/HeH X 101H mice not receiving $E_2$ than when these animals were OVX and $E_2$-treated (22).

Our finding that L-OVX animals demonstrated a marked LH response to NMA was surprising because in no case was an LH response to NMA obtained in young, middle-aged, or old S-OVX mice. NMA induced a significant rise in serum LH in the presence of the same physiological concentrations of $E_2$ regardless of animal age in L-OVX mice. These findings demonstrate that the presence of tonic, physiological levels of $E_2$ for 1 week, were in themselves insufficient to inhibit the NMA evoked GnRH secretion in the L-OVX animals. OVX for 3 months appears to be adequate for negating the $E_2$ inhibition of the NMA response, because young (OVX for 3 months), middle-aged (OVX for 4 months), and old (OVX for 20 months) animals all responded equally.

It has been proposed that the organizational effect of gonadal steroids on the hormone sensitive neural circuitry during the differentiation period of the hypothalamus allows steroidal activation of these circuits in adult life (40). In our study, L-OVX at puberty could have interfered with the gonadal steroid imprinting of the neural circuitry which mediates $E_2$ inhibition of NMA action. However, this is unlikely for the following reason. While the young and old animals were L-OVX at puberty, middle-aged mice were allowed to firmly establish ovarian cycles before L-OVX (i.e., OVX at 8 months of age). Thus, the length of time that the ovary has been absent, and not the age or the stage of peripubertal maturity is more likely to be the determining factor in the expression of $E_2$ inhibition of the LH response to NMA. It seems unlikely that organizational effects of gonadal steroids on hypothalamic neuronal circuitry are a factor in the different responses observed here.

Even though the old S-OVX animals had apparently been without significant endogenous estrogen for an extended time period, their response to NMA was quite different from young and old L-OVX mice. In fact, it was similar to young and middle-aged S-OVX animals. These data clearly indicate that the presence of the ovary for a sufficient time before administration of the excitatory amino acid in the ovariectomized mouse is necessary for $E_2$ inhibition of NMA evoked LH secretion. Intact old animals showed no evidence of biologically active estrogen as indicated by anestrus vaginal smears for 2 ± 0.5 months before experimentation, and their atrophic uteri. We cannot absolutely rule out the absence of $E_2$ since age-related impairment of $E_2$-induced changes in vaginal cytology have been reported in the C57BL/6J mice (41) and sufficient $E_2$ may be present to modulate gonadotropin secretion, but not vaginal cytology, in the old animals. It is also possible, however, that a non-$E_2$ ovarian factor may be modulating the $E_2$ inhibition of NMA stimulated GnRH secretion in aged female mice.

The ovarian influence on $E_2$ inhibition of the NMA-evoked GnRH response is most likely to be mediated through the neural targets of NMA. In support of this concept are the findings that NMA fails to induce LH secretion in lactating rats (42) and in rats whose endogenous proestrus LH surge has been blocked by the centrally acting drug, pentobarbitol (43). Although we consider it unlikely, we cannot completely eliminate the possibility that the difference between the LH responses to NMA in S-OVX and L-OVX mice is due, in part, to an alteration in the sensitivity of the pituitary to GnRH and/or changes in pituitary content. Altered pituitary sensitivity/content would most likely influence both the ability of the pituitary to respond, and the magnitude of the LH response to NMA. It is noteworthy that the LH responses to the two different doses of NMA in the L-OVX, estrogen primed mice (present study) are similar in amplitude to the LH responses to the low and high doses of GnRH administered to estrogen primed young and old S-OVX mice (see Ref. 10). Furthermore, the magnitude of circulating LH was similar in the S-OVX animals not given exogenous estrogen and the L-OVX animals that were given NMA (present

study). Thus, it seems unlikely that differences at the level of the gonadotrope alone can account for the all-or-none LH response to NMA observed in the estrogen-primed S-OVX and L-OVX mice.

In summary, this study has presented a model to differentiate the effects of aging on the responsiveness of the neuroendocrine hypothalamus from those associated with the ovary. We have demonstrated that $E_2$ inhibition of basal LH is not altered with age, and that long-term absence of the ovary does not alter the $E_2$ inhibition of basal GnRH secretion. However, ovarian activity does modulate the $E_2$ inhibition of NMA-stimulated GnRH secretion since long-term removal of the ovary prevented this inhibition. That the inhibitory effect is present in old, intact animals lacking estrous cycles that have also been deprived of the associated cyclical gonadal steroid secretion suggests the length of time from ovariectomy is an essential factor in the NMA response and supports the notion that the presence of the ovary is necessary to maintain this inhibition.

This work was supported by grants from the National Institutes of Health (RO1 AG07795 to M. M. M.) and the Fraser Endowment Fund of the Royal Victoria Hospital. D. J. is a recipient of doctoral studentship from Medical Research Council of Canada (ST-40237-AP004320). We want to thank Ms. I. Lekhtman for excellent technical help and Ms. Pat Smith and Ms. Reneé Lesage for performing RIAs for LH and $E_2$.

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