Effect of 17 β -estradiol on hypothalamic GnRH-II gene expression in the female rhesus macaque

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Abstract

The hypothalamus of rhesus macaques expresses two molecular forms of gonadotropin-releasing hormone (GnRH-I and GnRH-II). However, it is unclear whether these two neuropeptides play similar roles in the control of reproductive neuroendocrine function, especially in the context of positive and negative estrogen feedback. To address this issue, *in situ* hybridization histochemistry was used to compare the effect of 17 β -estradiol (E) on the expression of GnRH-I and GnRH-II mRNA in the medial basal hypothalamus (MBH) of adult female macaques. GnRH-I mRNA expression was found to be significantly ($P < 0.01$) more abundant in ovariectomized (ovx) animals compared with ovariectomized E-treated (ovx+E) animals. In marked contrast, GnRH-II mRNA expression was found to be significantly ($P < 0.05$) more abundant in ovx+E animals than in the ovx animals. To help elucidate how E exerts this stimulatory action on GnRH-II gene expression, hypothalamic sections were subsequently double labeled using a combination of immunohistochemistry for estrogen receptor (ER) α or β and *in situ* hybridization histochemistry for GnRH-II. Approximately 50% of the GnRH-II positive cells in the MBH were found to express ER β , but none expressed ER α . Taken together, these data give credence to a novel pathway by which E may control the primate neuroendocrine reproductive axis, one that involves stimulation of GnRH-II release via an ER β -mediated mechanism.

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Introduction

It has recently been demonstrated that a second form of gonadotropin-releasing hormone (GnRH), called chicken-II GnRH or GnRH-II, is expressed in the central nervous system of many mammalian species including some primates (Krey *et al.* 1975, Kasten *et al.* 1996, Dellovade *et al.* 1993, Chen *et al.* 1998, White *et al.* 1998, Urbanski *et al.* 1999). GnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂) differs by only three amino acids from the traditional mammalian form of GnRH (GnRH-I; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), but is encoded by a different gene (White *et al.* 1998) and is expressed by a distinct population of cells (Latimer *et al.* 2001). Moreover, GnRH-II has been detected in representative members of almost every extant vertebrate class, and its sequence is the most phylogenetically conserved of all the known GnRH forms (Miyamoto *et al.* 1984, Powell *et al.* 1986, Yu *et al.*

1988, Muske *et al.* 1990, Lovejoy *et al.* 1992, Krajewski *et al.* 2003). Although the physiological function of GnRH-II in vertebrates is unclear, its close structural similarity to GnRH-I suggests that it too may be a key component of the reproductive neuroendocrine axis.

Several issues remain unresolved in the field of primate reproductive neuroendocrinology, especially those pertaining to the control of puberty and the generation of the pre-ovulatory gonadotropin surge (Plant *et al.* 1978, Karsch 1987, Gore *et al.* 1993, Pau *et al.* 1993, Ma *et al.* 1994, Mitsushima *et al.* 1994, Kasten *et al.* 1996, Kasuya *et al.* 1999, El Majdoubi *et al.* 2000). Indeed, it is still equivocal whether enhanced GnRH-I release plays a fundamental role in triggering the pre-ovulatory gonadotropin surge. On the other hand, it has recently been demonstrated that GnRH-II is highly expressed in the rhesus macaque medial basal hypothalamus (MBH); this brain region has been implicated in both the pre-ovulatory surge (Spies

et al. 1977) and reproductive development (Khorram *et al.* 1988, Gore *et al.* 1993). Further, in gonad-intact rhesus macaques hypothalamic expression of GnRH-I mRNA does not increase significantly during pubertal development, whereas the expression of GnRH-II mRNA does, at least within the MBH (Latimer *et al.* 2000). Although this developmental increase in GnRH-II gene expression could play an important role in triggering puberty, it is equally plausible that the increase simply reflects a positive influence of estrogen on GnRH-II expression, and so represents a consequence of pubertal development rather than a cause. Nevertheless, the demonstration that GnRH-II is a potent stimulator of gonadotropin release *in vivo* (Lescheid *et al.* 1997, Densmore *et al.* 2003) further underscores the possibility that GnRH-II is physiologically involved in controlling the reproductive axis of primates.

In the present study, we used *in situ* hybridization histochemistry to compare GnRH-II mRNA levels in two groups of females: ovariectomized (ovx) and ovariectomized with 17 β -estradiol replacement (ovx+E). In addition, we used *in situ* hybridization histochemistry combined with immunohistochemistry to examine whether hypothalamic GnRH-II-expressing cells also express estrogen receptors, and thereby have the capacity to respond directly to changes in the sex-steroid environment. Preliminary findings have already been published as an abstract (Latimer & Urbanski 2002).

Materials and methods

Animals

This study was performed *in vitro* on hypothalami from adult (6–12 years old) female rhesus macaques (*Macaca mulatta*), obtained through the Oregon National Primate Research Center (ONPRC) Tissue Distribution Program; other tissues from these animals were used in unrelated studies. Prior to necropsy, animal care was provided in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and with approval of the ONPRC Animal Care and Use Committee. The animals were housed under controlled lighting (12 h light:12 h darkness) and temperature (23 $\pm$ 2 $^{\circ}$ C), and were provided with a diet consisting of Purina monkey chow and fresh fruit, with unlimited access to drinking water.

In Experiment 1, six ovariectomized animals were used to examine the influence of 17 β -estradiol (E) on GnRH-I and GnRH-II gene expression; the ovariectomies were performed at least 1 month prior to necropsy. Three of these animals served as untreated controls (ovx), while three received E replacement immediately after ovariectomy (ovx+E); in the latter group, silastic capsules containing crystallized E (Sigma) were implanted subcutaneously, as previously described (Gundlah *et al.* 2000) and this restored serum E concentrations (determined by RIA) to $\sim$ 60 pg/ml, which is within physiological parameters for the ovary-intact adult rhesus macaque (Karsch 1987). For reference, three gonad-intact females were also included in the study; at the time of necropsy, two of these were in the early-follicular phase of their menstrual cycle and one was in the luteal phase.

In Experiment 2, hypothalami from three adult gonad-intact females were used to examine whether GnRH-II cells express estrogen receptors. Based on detailed menstruation records, one of these animals was in the early-follicular phase of the menstrual cycle and the other two were in the luteal phase.

Tissue preparation

The animals were deeply anesthetized using ketamine/pentobarbital according to procedures established by the Panel on Euthanasia of the American Veterinary Society. Their brains were fixed by perfusing 1 l 0.9% saline through the ascending aorta followed by 6.5 l ice-cold 4% paraformaldehyde in 0.1 mol/l phosphate-buffered (pH 7.6) saline (0.9%, w/v). The hypothalami were blocked just rostral to the optic chiasm and rostral to the mammillary bodies, and immersed in fresh fixative for an additional 3 h (at 4 $^{\circ}$ C). They were then cryoprotected and sectioned (25 μ m) in the coronal plane using a freezing sliding microtome, as previously described (Latimer *et al.* 2001). For Experiment 1, the sections were immediately mounted on glass microscope slides (Fisherbrand SuperFrost/Plus, Fisher Scientific, Auburn, WA, USA), air-dried for 30 min, vacuum-dried overnight, and then stored at -85° C for later use in *in situ* hybridization histochemistry. For Experiment 2, the hypothalamic sections were stored free-floating in cryoprotectant at -20° C (Latimer *et al.* 2001), and subsequently used within 6 months for

combined immunohistochemistry and *in situ* hybridization histochemistry.

Experiment 1: effect of E on GnRH-I and GnRH-II gene expression

In situ hybridization histochemistry was used to determine the effect of E on GnRH gene expression. The procedure involved the use of previously validated ³⁵S-labeled antisense riboprobes to rhesus macaque GnRH-I mRNA or rhesus macaque GnRH-II mRNA (Ma *et al.* 1994, Urbanski *et al.* 1999, Latimer *et al.* 2001). These riboprobes were 224- or 430-nucleotides long respectively, and spanned the complete decapeptide-coding region as well as most of the GnRH-associated peptide (GAP) coding region. Because the GAP-coding region is unique for each GnRH precursor form, the probes specifically identified only those cells that express GnRH-I or GnRH-II mRNA respectively. The *in situ* hybridization histochemistry was performed, as described previously (Latimer *et al.* 2001), on a series of six coronal hypothalamic sections from each animal; the sections were collected at 200 µm intervals. To examine regional mRNA distribution at the cellular level, the sections were subsequently dehydrated using increasing concentrations of ethanol, defatted in xylenes for 1 h, and dipped in photographic emulsion (NTB-2, Eastman Kodak Co., Rochester, NY, USA) to induce silver grain deposition. They were exposed at 4 °C in a light-tight box for 12 days and then processed with Kodak developer (D-19) and fixer. Next, the sections were washed for 5 min in distilled water and dipped in thionine (Sigma) for 10 min, followed by two 5-min washes in water. The sections were then dehydrated with ethanol, cleared with xylenes, and finally coverslipped using DPX mounting medium.

A previous GnRH-II *in situ* hybridization study demonstrated that silver grain deposition accurately reflects optical density of autoradiographs (Latimer *et al.* 2000), but gives better spatial resolution; therefore, in the present study silver grain density was preferentially analyzed as a semi-quantitative measurement of GnRH expression under the different sex-steroid environments. The hypothalamic sections were examined under both bright-field and dark-field microscopy using a × 40 objective lens, and only cells that had an obvious round or fusiform silver grain deposition

pattern surrounding a thionine-stained nucleus were included in the analysis. The images were digitized using a Sony XC-77 CCD camera (Sony Corp. of America, Cypress, CA, USA) equipped with a 50-mm macro lens, and analyzed using the NIH Image computer program (version 1.59). Individual cells were defined using the freehand outlining tool, and both the silver grain density per cell (expressed as pixels per cell) and the total number of positive cells per section were determined. Unlike GnRH-II, GnRH-I is not expressed in distinct hypothalamic nuclei (Latimer *et al.* 2001); therefore, the mean level of GnRH-I expression across the whole hypothalamus was analyzed. In contrast, GnRH-II expression was analyzed for individual hypothalamic nuclei/regions. Two-way ANOVA was used to assess statistical differences between the treatment groups, and post-hoc analysis was performed using Newman–Keuls, when appropriate.

Experiment 2: expression of estrogen receptors in GnRH cells

We have previously observed estrogen receptor (ER)-β mRNA expression in regions of the macaque hypothalamus where GnRH-II mRNA is also expressed (Urbanski *et al.* 1999, Gundlach *et al.* 2000, Latimer *et al.* 2000, 2001). To examine whether there is co-localization of expression, we now collected 8–12 hypothalamic sections (25 µm thick) at ~200 µm intervals from each animal, and processed them for immunohistochemistry (for ERβ) followed by *in situ* hybridization histochemistry (for GnRH-II mRNA). The solutions for both of these procedures were made using RNase-free, diethyl pyrocarbonate-treated water. First, the sections were washed three times, 5 min each, with Tris buffer (0.05 M Tris, pH 7.6, containing 0.15 M sodium chloride). They were then incubated overnight at 4 °C with previously characterized antibodies (Russell *et al.* 2000, Saunders *et al.* 2000, Gundlach *et al.* 2001) to either ERα (Santa Cruz Biotech, Santa Cruz, CA, USA) or ERβ (a gift from Dr P T K Saunders) at 1:800 dilution in Tris buffer containing an RNase inhibitor (1 IU/ml; Promega). The sections were then washed three times, 5 min each, incubated in biotinylated goat anti-sheep or horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) at 1:1000 dilution in Tris buffer for 1 h at room

Figure 1 Effect of 17β -estradiol (E) replacement on GnRH gene expression in ovariectomized (ovx) rhesus macaques, as revealed by isotopic *in situ* hybridization histochemistry. Left column: Representative dark-field photomicrographs demonstrating a marked decrease in hypothalamic GnRH-I mRNA levels after E replacement. Right column: Representative dark-field photomicrographs demonstrating a marked increase after E replacement. Scale bar=15 μ m.

temperature, and again washed. To detect the immunoreactive signal, the sections were exposed to an avidin/biotin complex (Standard ABC kit, Vector Laboratories) for 1 h, washed three times in Tris buffer, 5 min each, and exposed to 3,3'-diaminobenzidine tetrachloride (DAB, 1 mg/ml Tris buffer; Sigma) for 10 min, concluding with 3 more washes. The sections were then mounted on glass microscope slides (Fisherbrand; Fisher Scientific), air-dried for 30 min, vacuum-dried overnight, and then stored at -85°C . The same sections were subsequently processed for *in situ* hybridization histochemistry using a ^{35}S -labeled antisense riboprobe against GnRH-II precursor cDNA, as described above, except that no thionine-counterstaining was performed. Similarly, they were dehydrated, defatted, dipped in photographic emulsion, and the pattern of silver grain deposition was examined under both bright-field and dark-field microscopy. Only cells that had an obvious round or fusiform silver grain deposition pattern, and a silver grain density greater than five times the background level, were counted and analyzed.

Results

E and GnRH-II gene expression

The hypothalami from ovx animals expressed significantly ($P<0.01$) more GnRH-I mRNA than the hypothalami from ovx+E animals (Fig. 1 left column, and Fig. 2A). This increased level of GnRH-I mRNA expression was reflected as an increase in the number of cells expressing silver grains at a level five times above background. The hypothalami from ovx animals also expressed significantly ($P<0.01$) more GnRH-I mRNA than hypothalami from the gonad-intact controls.

In marked contrast, the MBH from ovx+E animals expressed significantly ($P<0.05$) more GnRH-II mRNA than the MBH from ovx animals (Fig. 1 right column, and Fig. 2B); as with GnRH-I mRNA, the increased level of GnRH-II mRNA expression in the ovx+E animals was reflected as an increase in the total number of cells expressing silver grains at a level five times above background (Fig. 2B). It should be emphasized, however, that this differential expression of GnRH-II in response

Figure 2 Effect of E replacement on GnRH gene expression in ovx rhesus macaques. (A) Histogram depicting number of cells expressing GnRH-I mRNA in the hypothalamus of ovx and ovx + E animals, as revealed by *in situ* hybridization histochemistry (Fig. 1, left column). The number of detectable GnRH-I cells in the hypothalamus was markedly lower in the ovx + E animals than in the ovx animals. (B) Histogram depicting number of cells expressing GnRH-II mRNA in the MBH of ovx and ovx + E animals, as revealed by *in situ* hybridization histochemistry. The number of detectable GnRH-II cells in the MBH was markedly greater in the ovx + E animals than in the ovx animals. The *in situ* hybridization histochemistry was performed on a series of six coronal hypothalamic sections from each animal ($n=3/\text{group}$); the sections were collected at $\sim 200\ \mu\text{m}$ intervals. For reference, the mean number of GnRH-I- and GnRH-II-expressing cells detected in a heterogenous group of three gonad-intact animals (early-follicular phase and luteal phase) was 272 ± 74 and 41 ± 5 , respectively. ** $P < 0.01$, * $P < 0.05$.

to E was observed only within the MBH, and that no difference in GnRH-II mRNA expression was observed in the supraoptic nucleus (SON) and paraventricular nucleus (PVN), which served as negative controls (data not shown). Interestingly, the MBH from the heterogenous group of gonad-intact animals expressed significantly ($P < 0.01$) less GnRH-II mRNA than the MBH from the ovx+E animals.

Co-localization of estrogen receptors and GnRH-II mRNA

Digitized microscopic images were used to identify ER α or ER β expression (visualized as DAB precipitate) in GnRH-II-expressing cells (positively labeled cells showed a silver grain density that exceeded five times the background level). Approximately 50% of the GnRH-II-expressing cells in the MBH, and 40% in the PVN, showed immunoreactivity for ER β (Fig. 3, and Table 1). In contrast,

no regional overlap between ER β immunoreactivity and GnRH-II mRNA expression was evident in the SON. In addition, there was no detectable coexpression of GnRH-II and ER α in any hypothalamic region (data not shown).

Discussion

In a previous study, using ribonuclease protection assay, E was found to decrease GnRH-I gene expression in the hypothalamus of ovariectomized rhesus macaques (El Majdoubi *et al.* 1998). A similar result was obtained using *in situ* hybridization histochemistry, in the ovariectomized cynomolgus macaque (King *et al.* 1994). The results from the present *in situ* hybridization study corroborate both of these findings, by showing that E can markedly attenuate the post-ovariectomy rise in hypothalamic GnRH-I gene expression. Moreover, the results are in harmony with those from a

Figure 3 Representative photomicrographs of hypothalamic sections, double-labeled using a procedure that combined immunohistochemistry with *in situ* hybridization histochemistry. The sections were initially processed for ERβ immunohistochemistry and then for *in situ* hybridization histochemistry using a ³⁵S-labeled riboprobe to GnRH-II mRNA. ERβ-positive cells (showing nuclear staining) are indicated by arrowheads, and cells expressing GnRH-II mRNA are identified by densely clustered silver grains, indicated by arrows. Note the coexpression of ERβ and GnRH-II in the MBH and PVN but not in the SON. Scale bar=10 μm.

human postmortem study (Rance & Uswandi 1996) which showed that GnRH-I gene expression increases significantly in the MBH after menopause (i.e, when circulating E concentrations are extremely low or undetectable).

However, the present study is the first one to show that E can also effect GnRH-II gene expression in the MBH of a primate species. Surprisingly, unlike GnRH-I mRNA, GnRH-II mRNA levels were not suppressed after E replacement, instead they showed a marked *increase*. Indeed, the continuous delivery of exogenous E enhanced GnRH-II mRNA levels well above those observed in the gonad-intact animals (which were in the early-follicular and luteal phase of their menstrual cycle, and so were exposed to lower endogenous E levels). Taken together, the

findings from the GnRH-I and GnRH-II *in situ* hybridization studies give credence to the existence of two independent hypothalamic GnRH neuronal systems (Latimer *et al.* 2001), which differ markedly in their response to E. They also add support to a previous suggestion (Latimer *et al.* 2000) that functionally-distinct subpopulations of GnRH-II neurons may exist in the primate hypothalamus. Two observations, in particular, support this conclusion. First, E affected GnRH-II expression specifically in the MBH, but not in the PVN or SON, and secondly, ~50% of GnRH-II cells in the MBH and ~40% in the PVN expressed ERβ-immunoreactivity, whereas very little co-localization of GnRH-II and ERβ was evident in the SON. The latter observation suggests that the SON population of GnRH-II cells may function independently of estrogen signaling, although it does not rule out a possible indirect influence via inhibitory or stimulatory neurotransmitters. Conversely, although nearly half the GnRH-II cells in the PVN were found to express ERβ, GnRH-II gene expression in this nucleus did not appear to be affected by E; however, it is unknown whether E can effect the translation or release of GnRH-II from the PVN.

This demonstration that E exerts variable effects on different GnRH-II cell populations in the primate is reflective of the variable data that have been reported for other vertebrate species. For example, E did not alter midbrain GnRH-II expression in either the cichlid (*Oreochromis mossambicus*) or European silver eel (*Anguilla anguilla*)

Table 1 Co-expression of ERβ in hypothalamic GnRH-II cells

Hypothalamic nucleus	Number of ERβ-positive GnRH-II cells	Number of ERβ-negative GnRH-II cells
SON	19 (7%)	200 (93%)
PVN	28 (40%)	51 (60%)
MBH	41 (50%)	40 (50%)

GnRH-positive cells were identified by *in situ* hybridization histochemistry. Values represent the total number of GnRH-II cells that were immuno-positive or immuno-negative for ERβ in the examined sections (6 sections per animal). The respective percentages are shown in parentheses.

(Montero *et al.* 1995, Parhar *et al.* 1996). Also, the breeding season had no effect on whole-brain GnRH-II expression in the seabream (*Sparus aurata*) (Holland *et al.* 1998) or rockfish (*Sebastes rastrelliger*) (Collins *et al.* 2001), and stage of development had no effect on midbrain GnRH-II expression in rainbow trout (*Salmo gairdneri*) (Okuzawa *et al.* 1990). However, photostimulated turkey hens (*Meleagris gallopavo*) showed an increase in hypothalamic GnRH-II peptide (Rozenboim *et al.* 1993), and adult female turtles (*Trachemys scripta*) showed an increase in GnRH-II peptide relative to hatchling females (Tsai & Licht 1993). Also, African catfish (*Clarias gariepinus*) showed a developmental increase in GnRH-II immunoreactivity in the midbrain tegmentum (Dubois *et al.* 2001).

Interestingly, in the present study the most significant changes in GnRH-II gene expression were observed in the MBH, a region that has been implicated in several reproductive events including the control of puberty, pulsatile gonadotropin release, and possibly the pre-ovulatory gonadotropin surge (Atkinson *et al.* 1970, Nakai *et al.* 1978, Plant *et al.* 1978, Knobil *et al.* 1980, Caraty *et al.* 1998, Walsh & Clarke 1998, Kasuya *et al.* 1999, El Majdoubi *et al.* 2000, Plant 2001). This raises the provocative suggestion that GnRH-II may play a physiological role in the regulation of these events. Although this possibility remains to be substantiated experimentally, it gains support from the observation that GnRH-II is detectable in the systemic circulation of humans (Siler-Khodr & Grayson 2001), and that GnRH-II can dramatically stimulate gonadotropin release in rhesus macaques *in vivo* (Lescheid *et al.* 1997, Densmore & Urbanski 2003). In addition, we have previously reported that GnRH-II gene expression in the macaque MBH increases during development (Latimer *et al.* 2000). Although additional studies are needed to determine if this developmental increase is primarily driven by a sex-steroid independent mechanism, the present finding that E can stimulate GnRH-II gene expression in the MBH suggests that the developmental increase may simply be a response to the pubertal rise in circulating E concentrations (i.e., a *consequence* rather than a *cause* of puberty).

It is well accepted that E exerts negative feedback on pulsatile gonadotropin release, both at the hypothalamic and pituitary levels, and that E positive feedback is necessary for the pre-ovulatory

gonadotropin surge to occur (Atkinson *et al.* 1970, Yamaji *et al.* 1972, Karsch *et al.* 1973, Keye & Jaffe 1974, Spies & Norman 1975, Nakai *et al.* 1978, Ferin *et al.* 1979). However, the mechanisms underlying this apparent switch from negative to positive feedback are less clear. There are several convincing demonstrations that pituitary sensitivity to GnRH-I increases in primates following 36 h or more of E concentrations above 200 pg/ml (Yamaji *et al.* 1972, Ferin *et al.* 1979, King *et al.* 1995), and that a GnRH-I surge occurs prior to ovulation (Pau *et al.* 1993). Similarly, E negative feedback has also been demonstrated at the level of both the pituitary and the hypothalamus (Yamaji *et al.* 1972, Spies & Norman 1975, Ferin *et al.* 1979, Witkin *et al.* 1991, King *et al.* 1995), whether or not a GnRH-I surge is necessary for a gonadotropin surge to occur. However, the recent finding of a second estrogen receptor, ER β (Mosselman *et al.* 1996), supports the hypothesis that the negative feedback might occur through one system, via ER α , while a second system, employing ER β , may signal positive feedback (Ordog *et al.* 1998). Additional support for this hypothesis comes from the observation that E exerted a positive effect on a population of GnRH-II cells (in the MBH) that were shown to express ER β , whereas neither a negative effect nor ER α expression was observed in any of the GnRH-II cell populations. The present data that E treatment increased GnRH-II, but decreased GnRH-I, expression are also consistent with the hypothesis that E may exert alternate effects on different GnRH neural systems rather than switching from negative to positive feedback using a single system. Despite the provocative data suggesting that GnRH-II can exert an influence on some aspects of reproductive function, it remains to be proven that this influence is physiological. Furthermore, one cannot rule out the possibility that the primary physiological role of GnRH-II in primates involves an entirely different neuro-endocrine system, unrelated to reproduction.

In summary, the results from the present study suggest that E can directly exert a positive influence on GnRH-II gene expression in the MBH, whereas E primarily exerts an indirect negative influence on GnRH-I gene expression. This may help to explain why the net influence of E on the reproductive axis is negative when its concentration is below a certain threshold but positive when its concentration is higher; theoretically, a comprehensive

dose–response study should be able to identify a concentration of E (and duration of exposure) that tips the scales in favour of one GnRH control system over the other. Overall, the present results are consistent with the view that reproductive function in primates is controlled by functionally distinct GnRH neuronal pathways, and that these pathways respond differentially to changes in the sex-steroid environment.

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