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SEX STEROIDS AND THE CONTROL OF LHRH SECRETION

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Summary—Gonadal steroids are important hormonal signals that regulate the activity of LHRH synthesizing and releasing neurons. Aside from a direct effect through the feedback mechanisms exerted at hypothalamic and/or anterior pituitary level, gonadal steroids may modify the rhythmic LHRH release by modulating other systems affecting LHRH neurons.

1. In ovariectomized E_2-treated female rats, progesterone is able to evoke LHRH release from the perifused hypothalamus without affecting LH and FSH release.
2. Excitatory amino acids (EAA) and their related analogs (NMDA and kainate) are known to stimulate LH release in young rats. When tested in a perifusion system on hypothalamic and anterior pituitary tissues, they differentially stimulate the release of LHRH (NMDA) and of LH (KA); their effect on both structures is markedly reduced following orchidectomy.

It appears that gonadal steroids might exert a facilitatory action on the neurosecretory activity of LHRH neurons as well as a modulatory influence on the effect of EAA.

INTRODUCTION

Reproductive functions, both in females and males, are controlled by a rather intricated and refined network of endocrine signals; the hypothalamic hormone LHRH represents the primary element responsible for the modulation of gonadotropin secretion.

LHRH is synthesized in a relatively low number of neurons distributed in a scattered fashion in the septal and preoptic area as well as in the anterior hypothalamus. In humans and rodents, the LHRH gene encodes a 10 kDa prohormone protein which is processed to yield two biologically active peptides respectively of 10 and 56 amino acid residues, LHRH and GnRH-associated peptide (GAP) [1]. Secreted in a pulsatile way, LHRH regulates the number of pituitary LHRH receptors, gonadotroph responsiveness to LHRH action, as well as gonadotropin gene expression, biosynthesis and release [2–5].

The activity of LHRH synthesizing and releasing neurons is regulated by several neural circuitries whose anatomical and functional integration with LHRH neurons is essential for the acquisition of hypothalamic reproductive competence. Also gonadal secretion obviously exerts a profound impact on brain regulation of reproductive phenomena. Gonadal steroids participate in an intricate system of feedback loops controlling the hypothalamo–pituitary–gonadal axis. Available evidence, however, suggests that this feedback control on LHRH secretion is not simply exerted through direct effects of the steroids on LHRH secreting neurons. Both autoradiographic and immunocytochemical studies have failed to demonstrate measurable concentrations of [^{3}H]estradiol in LHRH-immunoreactive perikarya in the rat brain, suggesting that these neurons do not themselves contain estrogen receptors [6]. In addition, a variety of experimental manipulations that cause disruption of cyclic ovarian function do not significantly affect the ultrastructure or distribution of the LHRH-immunoreactive neurons [7]. These observations are consistent with the hypothesis that the effects of gonadal steroids on LHRH release are mainly indirect and primarily mediated through inputs from other steroid-sensitive neuronal systems.

The present report will summarize the data obtained in the authors' laboratory on the role of sex steroids on LHRH and LH release, as well as on their modulatory influences on the effect of other stimuli. In particular, the paper will describe: (a) the *in vivo* and *in vitro* action

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of estrogen and progesterone on LHRH-LH release; and (b) the effect of gonadal steroids on the hypothalamic and pituitary responses to excitatory amino acids.

ROLE OF PROGESTERONE ON LHRH AND LH RELEASE

In the female rat, estrogen and progesterone may exert both a positive and a negative feedback effect. Ovariectomy results in elevated secretion of LH and FSH, which can be suppressed by estrogen replacement. On the other hand, the role of estrogens in triggering the preovulatory surge of gonadotropins has been demonstrated by several lines of evidence [8, 9]. As far as progesterone is concerned, its biphasic effect on gonadotropin secretion is related to the time of administration during the ovulatory cycle. When injected late in the cycle, progesterone has been found to advance the ovulatory LH surge, while, on the contrary, administration of the steroid early in the estrous cycle leads to inhibition of the expected LH surge during the afternoon of proestrus [10, 11].

Ovarian steroids may interact with gonadotropic control mechanisms at multiple sites. Estrogens, as well as progesterone, might change the responsiveness of pituitary gonadotrophs to LHRH [12–14] and modify synthesis, storage and/or release of the decapeptide at hypothalamic level [15, 16].

In our *in vivo* studies, adult female rats have been ovariectomized regardless of the phase of the estrous cycle and subcutaneously injected with ethiny estradiol (0.4 μg/rat/day) for 5 days. On the morning of the fifth day they have been injected with progesterone (100 μg) and sacrificed 6 h later. Figure 1 shows that, in such

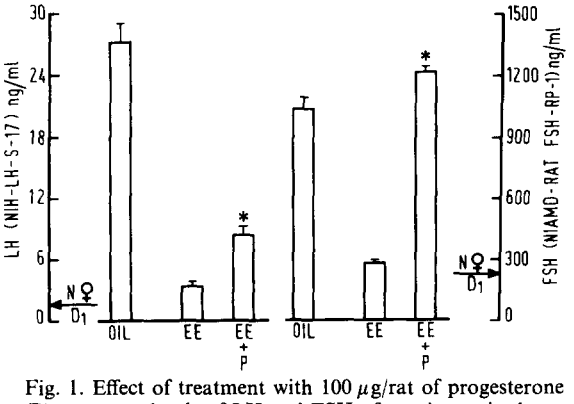

Fig. 1. Effect of treatment with 100 μg/rat of progesterone (P) on serum levels of LH and FSH of ovariectomized rats treated for 5 days with 0.4 μg of ethinyl estradiol (EE) and sacrificed 6 h after progestagen administration.

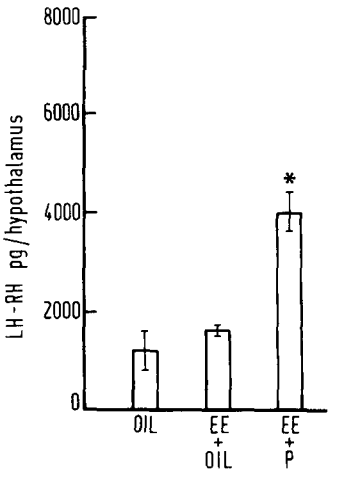

Fig. 2. Effect of treatment with 100 μg/rat of progesterone (P) on hypothalamic content of LHRH of ovariectomized rats treated for 5 days with 0.4 μg of ethinyl estradiol (EE) and sacrificed 6 h after progestagen administration.

experimental animals, LH and FSH levels are increased by the administration of progesterone; also hypothalamic LHRH content is markedly enhanced by the administration of progesterone (Fig. 2). These results may be interpreted as suggesting that progesterone triggers the release of LHRH from the hypothalamus to the portal vessels and thus stimulates the release of LH from the anterior pituitary. This hypothesis is supported by the demonstration of increased concentrations of LHRH in the portal vessels of proestrous [17] and ovariectomized steroid-treated rats [18]. These observations prompted us to examine the effect of progesterone on the release of LHRH from the mediobasal hypothalamic–preoptic area (MBH–POA) and of LH and FSH from the anterior pituitary using an *in vitro* perifusion technique. The tissues were obtained from ovariectomized rats implanted for 5 days with silastic capsules containing estradiol-17β (235 μg/ml). MBH-POA and anterior pituitary collected from the same animal have been perifused in two parallel chambers. Figure 3 shows the effect of intermittent superfusions with progesterone (5 min pulses, separated by 20 min interval) at two different doses (10 and 25 ng/ml). On the left side of the figure the dynamic secretion of LHRH from the MBH-POA (upper left panel) and of LH and FSH from the anterior pituitary are represented (lower left panels). On the right side of the figure, the responses to different stimuli have been expressed as Δ in picograms of LHRH or in ng of LH and FSH released, with Δ representing the total amount of hormones released during the whole secretory response to each stimulus minus the amount of hormone released

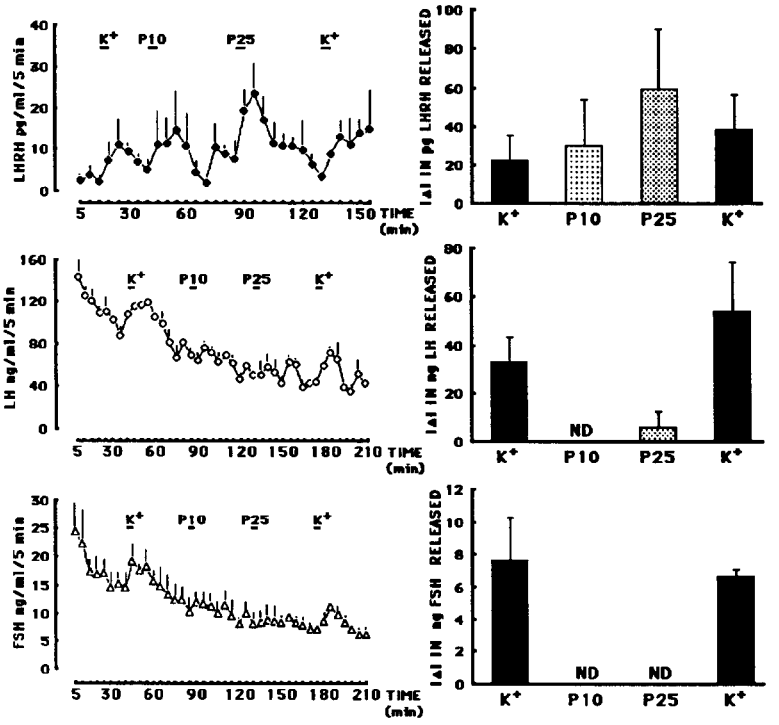

Fig. 3. Release of LHRH, LH and FSH in response to pulses of K^+ (56 mM) and Progesterone (P, 10 and 25 ng/ml) from perifused hypothalamus and anterior pituitary of ovariectomized rats implanted with 17β-estradiol since 5 days.

over the same period of time under basal conditions. Both pulses of progesterone induce LHRH secretion, with an effect that occurs immediately, and does not appear to be related to the dose of the steroid. At the beginning and at the end of the experimental perifusion period, two pulses of K^+ (56 mM) have been applied which resulted in the expected release of LHRH due to the depolarizing action of K^+.

On the contrary, no changes in LH and FSH release from the anterior pituitary of the same rat have been observed following perifusion with progesterone. The pituitary tissue was, however, viable since it responded to the K^+ stimulations with bursts of both LH and FSH.

The data indicate first of all that the hypothalamus is the main site of action for the positive feedback effect of progesterone on gonadotropin secretion. The amplitude of the release of LHRH induced by progesterone is comparable to that obtained after K^+ stimulation and, like that to K^+, the response is almost immediate and transient. Contrary to what has been reported by other authors [19, 20] no latency has been observed in the response to progesterone. This result, together with the similarity with the effects of K^+, is suggestive of an action of progesterone at the level of the cell membrane inducing a signal triggering intracellular mechanisms initiating the release of the decapeptide [21, 22]. The mechanism of the modulatory effect of progesterone on LHRH secretion observed in these studies is clearly not genomic, due to the very short time required to manifest. However, we cannot exclude that at later intervals an interaction of the steroid with cytosolic receptors might occur leading to a subsequent alteration of either gene transcription or biosynthetic processing of LHRH from its precursor. It must be pointed out, however, that recently discrepant results have been obtained by different groups on the effect of progesterone and other sex steroids on LHRH gene expression measuring LHRH mRNA levels, either by Northern blotting or by *in situ* hybridization [23].

EFFECT OF SEX STEROIDS ON THE ACTION OF EXCITATORY AMINO ACIDS, ON LHRH AND LH RELEASE

Excitatory amino acids (EAA) and in particular glutamate (Glu) and aspartate (Asp) act as neurotransmitters in many areas of the central nervous system including the hypothalamus [24, 25]. Present in large amounts at the nerve terminals they are released into the synaptic cleft following appropriate stimuli and exert

their excitatory actions through the binding to specific receptors. Actually at least 3 subtypes of receptors have been identified on the basis of their binding affinities utilizing as ligands different analogs, namely *N*-methyl-D-aspartate (NMDA), kainate (KA) and quisqualate. The three subtypes of receptors have thus been identified as NMDA-receptors, and non-NMDA receptors, the latter group being further subdivided into KA and quisqualate receptors. It has been proposed that at low systemic doses EAA and their analogs, NMDA and KA, excite neurons in the arcuate nucleus of the hypothalamus while at higher doses they may be neurotoxic [26]. Recent evidence suggests that EAA and related agonists may play a role in the control of neuroendocrine functions. Several reports indicate that NMDA and KA activate hypothalamic structures involved in the control of anterior pituitary function. *In vivo* studies have shown an increase in LH secretion in the rat following administration of either EAA or their related agonists, this effect being maximum between 20 and 40 days of age [27, 28]. On the basis of these observations, it has been assumed that NMDA can be a useful neuroendocrine probe to elucidate central neurotransmitter system controlling and modulating LH secretion. Since by *in vitro* experiments it has been shown that NMDA does not seem to affect the release of LH acting directly at the anterior pituitary level and since NMDA-induced gonadotropin release is abolished by prior treatment with an LHRH receptor antagonist, it has been suggested that the action of NMDA might be exerted at hypothalamic level, through the stimulation of LHRH release [29]. In order to directly clarify the site (hypothalamus and/or pituitary) at which EAA exert their effect on gonadotropin secretion, the following *in vitro* experiments have been performed.

In these studies the hypothalamus and the anterior pituitary obtained from the same animal (normal male, 45–55 day old rats) have been perifused in two separate chambers either in the absence or in the presence of two amino acid analogs (NMDA and KA). The levels of LHRH and LH have been measured in the effluent obtained from the respective chambers. To test the modulatory action of sex steroids on the sensitivity of the hypothalamus and of the anterior pituitary to EAA, the same experiments have been performed utilizing tissues obtained from animals of the same age and castrated since one week old. In order to ascertain that the effect of EAA on LHRH secretion is not aspecifically due to artefacts, the response of the

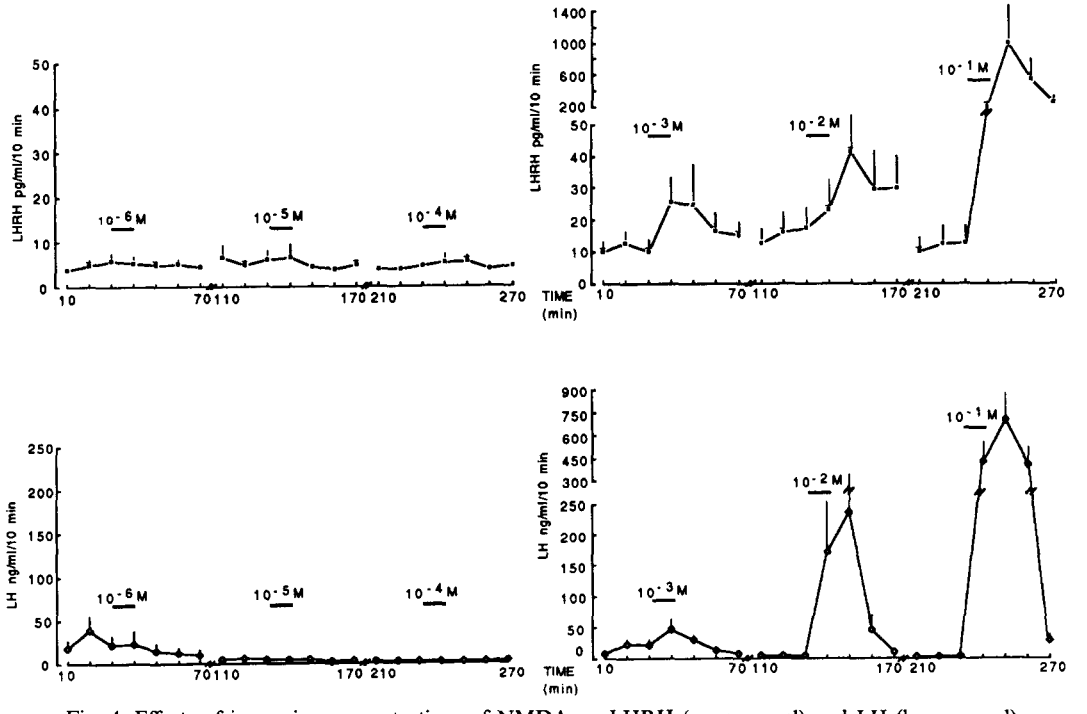

Fig. 4. Effects of increasing concentrations of NMDA on LHRH (upper panel) and LH (lower panel) release from perifused hypothalamus and anterior pituitary of normal male rats. Means ± SEM of 4 experiments.

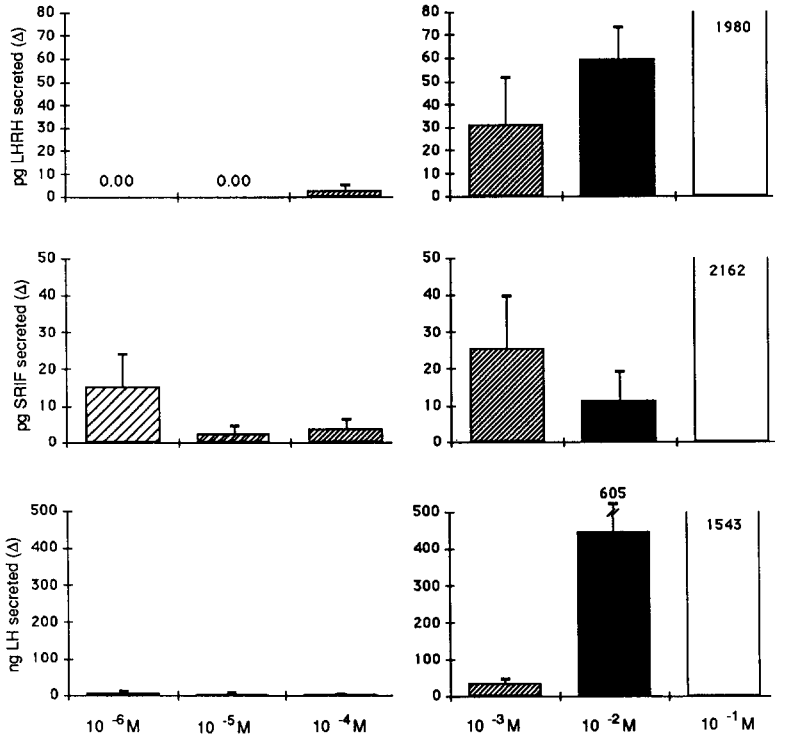

Fig. 5. Total release of LHRH, SRIF and LH induced by increasing NMDA concentrations from perifused hypothalamus and anterior pituitary of normal male rats. Values are expressed as Δ; means ± SEM of 4 experiments.

hypothalamus have been checked also in terms of somatostatin (SRIF) release.

The results obtained by submitting the perifused hypothalami and anterior pituitaries of normal young male rats to pulses of increasing concentrations of NMDA (10^{-6}–10^{-1} M) are reported in Fig. 4 (dynamic release of LHRH and LH) and in Fig. 5 (Δ in picograms of LHRH and in nanograms of LH secreted under the stimuli). At the concentrations of 10^{-3} and 10^{-2} M NMDA stimulates LHRH release, the effect of 10^{-2} M appearing as more prominent. On the contrary, the concentration of 10^{-3} M is more effective than the concentration of 10^{-2} M on SRIF release (Fig. 5). When tested on the anterior pituitary (Fig. 5), NMDA proved to be very effective in inducing LH release only when used at the concentration of 10^{-2} M. The effects obtained both at pituitary and at hypothalamic level when the concentration of 10^{-1} M has been used may probably be considered as a non-specific result, which however provides strong evidence for the viability of the tissues. Orchidectomy, performed 1 week prior to sacrifice completely abolishes the response obtained with a 10^{-3} M pulse of NMDA on LHRH and SRIF (Fig. 6); the stimulatory effect of the 10^{-2} M dose persists even if reduced on LHRH but is totally absent on SRIF. The effect of NMDA (10^{-2} M) at anterior pituitary level on LH secretion is markedly reduced following castration.

Kainate applied in pulses of 10^{-6}–5×10^{-2} M concentrations (Figs 7 and 8) to the hypothalamus of normal young male rats, has very little effect on LHRH but stimulates SRIF release at the concentrations of 10^{-3} and 10^{-2} M. At anterior pituitary level the lowest dose (10^{-6} M) of the compound is highly effective in inducing LH secretion. The effect disappears at the 10^{-5}–10^{-4} M concentrations, and reappears at 10^{-3} and 10^{-2} M. Also in castrated animals (Fig. 9) KA remains without effect on LHRH secretion; on the contrary, castration reduces the effect on SRIF; in fact only the 10^{-2} M concentration of KA is still able to stimulate somatostatin release after orchidectomy. As in the case of NMDA, the anterior pituitary of castrated rats is much less responsive to KA in terms of LH release.

The present data, while confirming a stimulatory action of both NMDA and KA on LHRH release, gives some additional insight on their neuroendocrine mode of action. First of all, some differences are apparent on the effects of the two EAA analogs on the release of

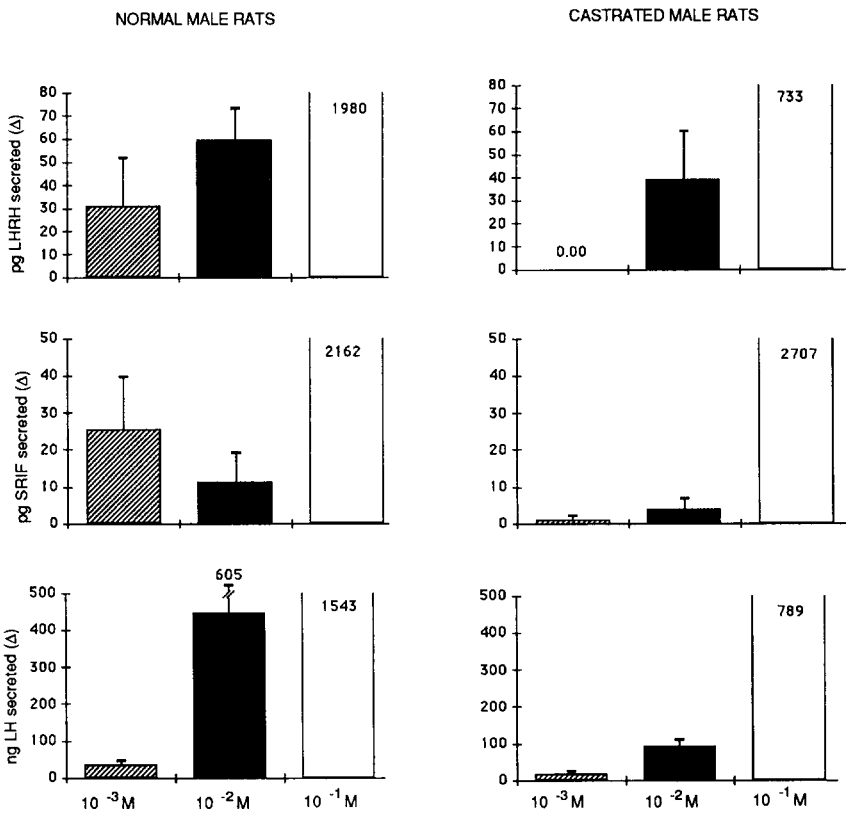

Fig. 6. Total release of LHRH, SRIF and LH induced by increasing NMDA concentrations from perifused hypothalamus and anterior pituitary of normal and castrated male rats. Values are expressed as Δ; means ± SEM of 4 experiments.

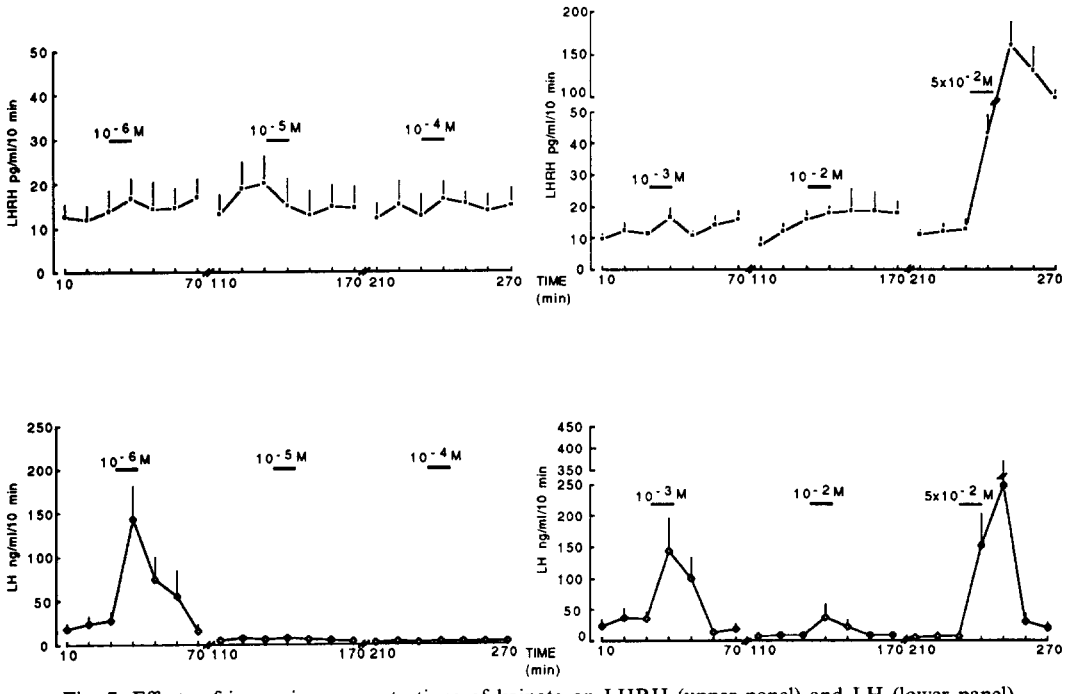

Fig. 7. Effects of increasing concentrations of kainate on LHRH (upper panel) and LH (lower panel) release from perifused hypothalamus and anterior pituitary of normal male rats. Means ± SEM of 4 experiments.

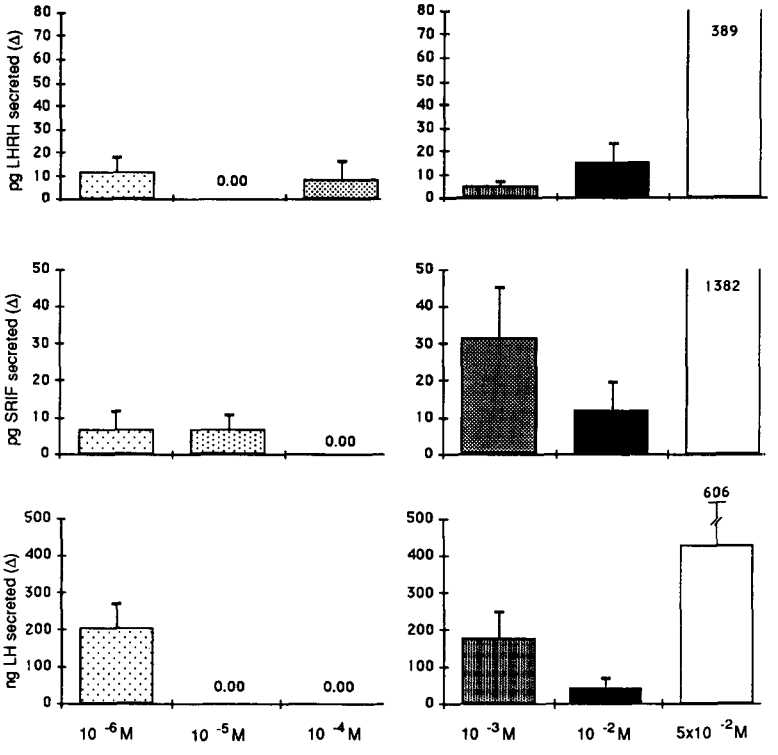

Fig. 8. Total release of LHRH, SRIF and LH induced by increasing kainate concentrations from perifused hypothalamus and anterior pituitary of normal male rats. Values are expressed as Δ; means ± SEM of 4 experiments.

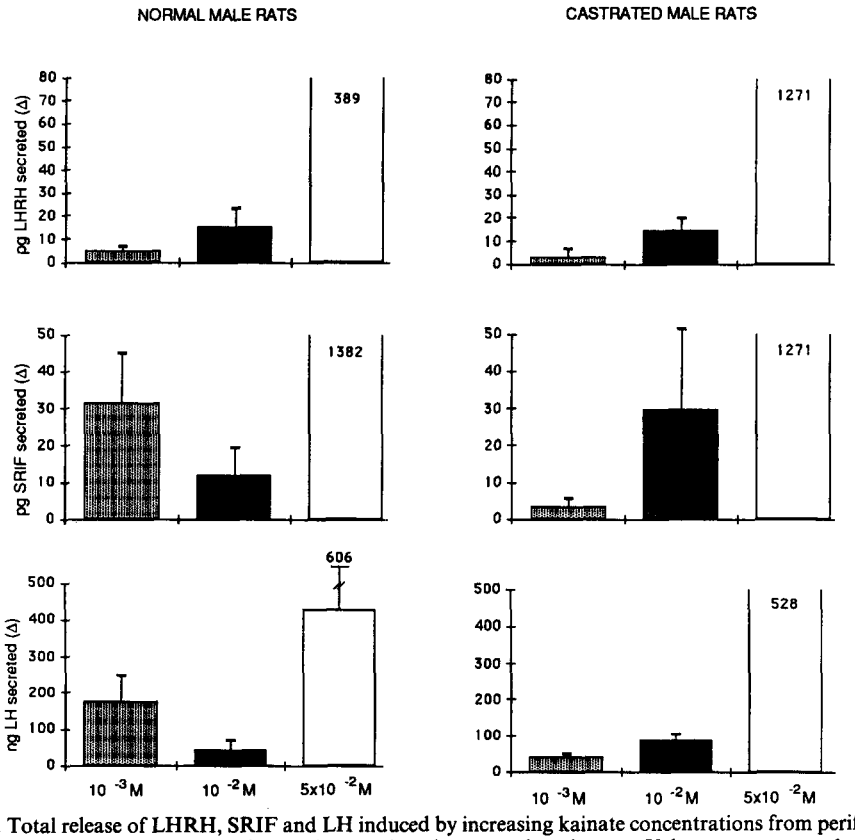

Fig. 9. Total release of LHRH, SRIF and LH induced by increasing kainate concentrations from perifused hypothalamus and anterior pituitary of normal and castrated male rats. Values are expressed as Δ; means ± SEM of 4 experiments.

LHRH and SRIF; NMDA appears to be more potent in releasing LHRH, while KA seems to be more effective in releasing SRIF. Moreover, castration decreases the effects of NMDA on both LHRH and SRIF release, while the effect of kainic acid on SRIF release appears to be less affected. One of the most relevant findings of this series of investigations is the significant effect exerted by NMDA, and particularly by kainate, at pituitary level. This has not been previously reported by other authors using *in vitro* techniques. The difference may reside in the fact that the present experiments have been performed using a perifusion system rather than static conditions. On the basis of *in vivo* studies utilizing LHRH antagonists, it has been postulated that the EAA act exclusively at hypothalamic level through the stimulation of LHRH release [30]. The present data do not exclude such a possibility but adds the new information that, in particular conditions, the EAA may also act directly on the anterior pituitary. It is interesting to underline that the direct effects of NMDA and of kainate on LH secretion is abolished in the absence of androgens; further work will clarify whether this effect is due to a modulatory influence of sex steroids on EAA receptors.

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REFERENCES

1. Adelman J. P., Mason A. J., Hayflick J. S. and Seeburg P. H.: Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 179–183.
2. Marshall J. C. and Kelch R. P.: Gonadotropin-releasing hormone: role of pulsatile secretion in the regulation of reproduction. *New Engl. J. Med.* **315** (1986) 1459–1468.
3. Andrews W. W., Maurer R. and Conn P. M.: Stimulation of rat luteinizing hormone-beta messenger RNA levels by gonadotropin-releasing hormone. *J. Biol. Chem.* **263** (1988) 13755–13761.
4. Hubert J. F., Simard J., Gagné B., Barden N. and Labrie F.: Effect of LHRH and D-Trp^8, des-Gly-NH$^{10}_2$ LHRH ethylamide on alfa-subunit and LHbeta mRNA levels in rat anterior pituitary cells in culture. *Molec. Endocr.* **2** (1988) 521–527.
5. Wierman M. E., Rivier J. E. and Wang C.: Gonadotropin-releasing hormone-dependent regulation of gonadotropin subunit messenger ribonucleic acid levels in the rat. *Endocrinology* **124** (1989) 272–278.
6. Shivers B., Harlan R., Morrel J. and Pfaff D.: Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. *Nature* **304** (1983) 345–347.
7. King J., Tobet S., Snavely F. and Arimura A.: The LHRH system in normal and neonatally androgenized female rats. *Peptides* **1** (1980) 85–100.
8. Broom G. M. and Schwartz N. B.: Acute changes in the estrus cycle following ovariectomy in the golden hamster. *Neuroendocrinology* **3** (1968) 366–377.
9. Legan S. J., Conn G. A. and Karsch F. J.: Role of estrogen as initiator of daily LH surges in the ovariectomized rat. *Endocrinology* **96** (1975) 50–56.
10. Everett J. W.: Progesterone and estrogen in the experimental control of ovulation time and other features of estrous cycle in the rat. *Endocrinology* **43** (1948) 389–405.
11. Caligaris L., Astrada J. J. and Taleisnik S.: Biphasic effect of progesterone on the release of gonadotrophin in rats. *Endocrinology* **89** (1971) 331–344.
12. Negro-Vilar A., Orias R. and McCann S. M.: Evidence for a pituitary site of action for the acute inhibition of LH release by estrogen in the rat. *Endocrinology* **92** (1973) 1680–1684.
13. Gordon J. H. and Reichlin S.: Changes in pituitary responsiveness to luteinizing hormone-releasing factor during the rat estrous cycle. *Endocrinology* **94** (1974) 974–987.
14. Drouin J. and Labrie F.: Interactions between 17beta-estradiol and progesterone in the control of luteinizing hormone and follicle stimulating hormone release in rat anterior pituitary cells in culture. *Endocrinology* **108** (1981) 52–57.
15. De Paolo L. V., Ojeda S. R., Negro-Vilar A. and McCann S. M.: Alterations in the responsiveness of median eminence luteinizing hormone-releasing hormone nerve terminals to norepinephrine and prostaglandin E_2 *in vitro* during the rat estrous cycle. *Endocrinology* **110** (1982) 1999–2005.
16. Rance N., Wise P. M. and Barraclough C. A.: Negative feedback effects of progesterone correlated with changes in hypothalamic norepinephrine and dopamine turnover rates, median eminence luteinizing hormone-releasing hormone and peripheral plasma gonadotropin. *Endocrinology* **108** (1981) 2194–2199.
17. Sarkar D. K. and Fink G.: Effects of gonadal steroids on output of luteinizing hormone-releasing factor into pituitary stalk blood in the female rat. *J. Endocr.* **80** (1979) 303–313.
18. Sarkar D. K. and Fink G.: Luteinizing hormone-releasing factor in pituitary stalk plasma from long-term ovariectomized rats: effects of steroids. *J. Endocr.* **86** (1980) 511–524.
19. Kim K. and Ramirez V. D.: *In vitro* progesterone stimulates the release of luteinizing hormone-releasing hormone from superfused hypothalamic tissue from ovariectomized estradiol-primed prepuberal rats. *Endocrinology* **111** (1982) 750–756.
20. Drouva S. V., Laplante E. and Kordon C.: Progesterone-induced LHRH release *in vitro* is an estrogen—as well as Ca^{++}—and calmodulin-dependent secretory process. *Neuroendocrinology* **40** (1985) 325–331.
21. Baulieu E. E.: Current approaches to steroid hormone-cell interactions. In *Central Regulation of the Endocrine System* (Edited by K. Fuxe, T. Hökfelt and R. Luft). Plenum Press, New York (1979) pp. 239–260.
22. Towle A. C. and Sze P. Y.: Steroid binding to synaptic plasma membrane: differential binding of glucocorticoids and gonadal steroid. *J. Steroid Biochem.* **18** (1983) 135–143.
23. Toranzo D., Dupont E., Simard J., Labrie C., Couet J., Labrie F. and Pelletier G.: Regulation of pro-gonadotropin-releasing hormone gene expression by sex steroids in the brain of male and female rats. *Molec. Endocr.* **3** (1989) 1746–1756.
24. Watkins J. C. and Evans R. H.: Excitatory aminoacid transmitters. *A. Rev. Pharmac. Toxic.* **21** (1981) 165–176.
25. Onley J. W. and Price M. T.: Excitotoxic aminoacids as neuroendocrine probes. In *Kainic Acid as a Tool in*

Neurobiology (Edited by E. G. McGeer, J. W. Olney and P. L. McGeer). Raven Press, New York (1978) pp. 239–263.
26. Ondo J. G., Pass K. A. and Baldwin R.: The effects of neuronally active amino acids on pituitary gonadotropin secretion. *Neuroendocrinology* **21** (1976) 79–87.
27. Arslan M., Pohl C. R. and Plant T. M.: DL-2-Amino -5-phosphonopentatoic acid, a specific *N*-methyl-D-aspartic acid receptor antagonist, suppresses LH release in the rat. *Neuroendocrinology* **47** (1988) 465–468.
28. Schainker B. A. and Cicero T. J.: Acute central stimulation of luteinizing hormone by parenterally administered *N*-methyl-D,L-aspartic acid in the male rat. *Brain Res.* **184** (1980) 425–437.
29. Bourguignon J. P., Gerard A., Mathieu J., Simons J. and Franchimont P.: Pulsatile release of gonadotropin-releasing hormone from hypothalamic explants is restrained by blockade of *N*-methyl-D,L-aspartate receptors. *Endocrinology* **125** (1989) 1090–1096.
30. Donoso A. O., Lopez F. J. and Negro-Vilar A.: Glutamate receptors of the non-*N*-methyl-D-aspartic acid type mediate the increase in luteinizing hormone-releasing hormone release by excitatory amino acids "*in vitro*". *Endocrinology* **126** (1990) 414–420.