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FETAL LEYDIG CELL CULTURE—AN *IN VITRO* SYSTEM FOR THE STUDY OF TROPHIC HORMONE AND GnRH RECEPTORS AND ACTIONS

MARIA L. DUFAU and GAIL F. KNOX
Molecular Endocrinology Section, Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205, U.S.A.

Summary—In fetal and neonatal rat Leydig cells cultured in the presence of LH, gonadotropin and GnRH receptors and acute testosterone responses to hCG, were maintained for up to 78 days. Addition of GnRH agonists markedly inhibited steroid production in LH-treated cultures, and abolished the acute testosterone response to hCG. GnRH receptors were not detectable in fetal testes but were present post-natally and increased markedly with age. In cultured fetal testis, GnRH receptors were detected on the third day, and were increased by exposure to GnRH agonists. In LH-treated cultures, GnRH sites were reduced by about 50% and did not increase during incubation with GnRH agonists. LH supported 17α-hydroxylase/17-20 desmolase activities and pregnenolone formation were observed in short (1–4 days) and long-term cultures. Also, LH stimulation of 3β-hydroxysteroid dehydrogenase was observed by histochemical studies at 7 days of culture. GnRH agonists inhibited LH dependent steroid production in a dose-dependent fashion and abolished the acute testosterone response to human chorionic gonadotropin. The major component of the steroid inhibitory effect of GnRH agonist occurs beyond cAMP production. A distal lesion of the microsomal enzymes of the androgen pathway is largely responsible for the GnRH-induced decreases in LH-supported androgen production. The expression of functional GnRH receptors during culture and their suppression by LH suggest that pituitary gonadotropins exert a tonic inhibitory effect upon testicular GnRH receptors. The presence of functional GnRH receptors and inhibitory actions in cultured fetal and neonatal Leydig cells indicates that GnRH-related peptides can influence the actions of gonadotropins on the fetal Leydig cell population.

INTRODUCTION

GnRH and its agonist analogues caused inhibitory changes in testicular function [1–5] that are due to direct effects via specific GnRH receptors present in the Leydig cell [3, 4, 6–9]. Such inhibitory effects of GnRH on androgen production were initially demonstrated *in vivo* during treatment of hypophysectomized rats [3]. In contrast the acute inhibitory actions of GnRH observed *in vivo* in intact animals were mainly due to steroidogenic lesions caused by LH action in the testis through a short loop feed-back control of microsomal enzymes of the androgen pathway exerted via the nuclear actions of estrogen [10]. In addition, a more prolonged treatment of intact animals with the peptide more profoundly reduced gonadal function by causing pituitary desensitization [4, 11], and in this case the direct inhibitory effects of GnRH in the gonads presumable are also present. Some of the direct early effects of GnRH in the testis have been demonstrated in acute culture of adult Leydig cells. These have been mainly stimulatory [12, 13], since the inhibitory effects are not readily demonstrable during short-term incubations, but have been observed in cultured interstitial cells from hyphysectomized animals and fetal cultures [1, 5, 14, 15]. The cultured fetal Leydig cells have considerable potential for long-term studies and for elucidation of molecular mechanism of GnRH and trophic hormone action [15]. In this paper we will summarize our recent studies using these cultures and review current views of GnRH action in the Leydig cells.

GnRH RECEPTORS: BINDING CHARACTERISTICS AND REGULATION

GnRH binding to adult Leydig cells

There is now a general recognition of the existence of GnRH receptor sites in the rat testis [3, 4, 6, 9]. However, the physiological significance of the presence of these receptors in the rat is still unclear, since the receptor and direct actions of GnRH have not been readily demonstrable in the human [16], and mouse testes [13].

Furthermore, the levels of this peptide in the circulation are below the limit of detection of the present methods and are unlikely to exert direct gonadal effects. Although testicular GnRH-like activity has been suggested to be a secretory product of Sertoli cells [17, 18], the isolation and characterization of this or related peptides have not yet been accomplished. Furthermore, recent studies have demonstrated the presence of significant GnRH peptidase activity in the testis, raising the possibility that tracer degradation could cause artifactual estimates of GnRH-like activity in radioimmunoassay or radioreceptor assays employed to monitor testicular extracts in several previous studies. Taking into account this enzymatic activity, and after modification of the

extraction procedure, the testicular content of GnRH-like activity was estimated to be less of 15 pg per rat testis [19].

Most of the information available on the binding properties of testicular GnRH receptors in the rat has been derived from studies using whole testis homogenates, interstitial cell particles and crude Leydig cells [3, 4, 6–9]. Such studies have suggested that testicular GnRH receptors are located on the Leydig cell surface. In more recent studies, we have assessed the binding of GnRH-A to Leydig cells purified by centrifugal elutriation [20]. Analysis of Scatchard plots and saturation curves showed a single class of binding sites with K_a of $2.4 \times 10^9\ M^{-1}$ and a binding capacity of 3,000 sites per cell (Fig. 1). The binding affinity observed in our studies [21] was similar to those reported in crude Leydig cells and cell membranes (Table 1). Also, the concentration of receptors expressed per testis was of the same magnitude of those derived in earlier studies from interstitital tissue preparations and from cells obtained by Percoll purification [13]. The above findings indicated that the process of Leydig cells purification (i.e. collagenase dispersion followed by elutriation) did not affect the binding properties of the GnRH receptor.

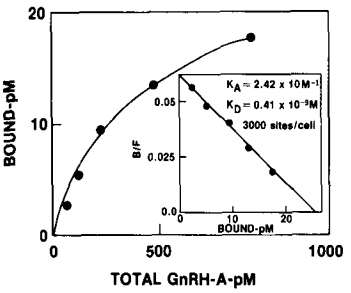

Fig. 1. Saturation curve and Scatchard analysis (inset) of GnRH analog ([*D*-Ala^6]des-Gly^{10}-GnRH-*N*-ethylamide) binding to adult Leydig cells purified by centrifugal elutriation [20]. Cells ($1–5 \times 10^6$) from a pool of fractions 8–12 were incubated at 4°C for 80 min with increasing concentrations of [^{125}I]GnRH analog as previously described [27]. Results are the mean of three closely agreeing experiments.

Ontogeny of GnRH receptors and regulation of receptors by homologous and heterologous hormone

GnRH receptors were not detectable in homogenates of acutely excised 20.5 day fetal testes or in freshly prepared fetal Leydig cells (20.5 day), but detectable from 3 days to 70 days of culture, the latest time examined [15, 22]. These receptors were also readily detectable postnatally in the testes of 5-day old rats and increased markedly during maturation at 30 and 40 days (Fig. 2). The equilibrium association constant (K_a) derived by Scatchard analysis of the GnRH-A [D-Ala^6], des-Gly^{10}-GnRH-*N*-ethylamide) binding data was $6.8 \pm 1.7 \times 10^9\ M^{-1}$ in the 5-day old rat testes and was similar in fetal testes cultured for 4 days. These values are also close to those reported in adult Leydig cells [21].

GnRH receptor concentration increased by 40 and 160% in cultures exposed to GnRH and GnRH-A ($P < 0.01$). Incubation of GnRH or GnRH-A treated fetal cultures [15] with ovine LH reduced the GnRH receptors to below control levels ($P < 0.01$) and addition of oLH to control cultures reduced receptors by 50% ($P < 0.01$), [15], Fig. 3. In other experiments we have also demonstrated that GnRH treatment did not significantly affect the fetal LH receptors [15, 22].

Of particular interest was the finding that GnRH receptors were not detectable in particulate fractions of freshly dissected fetal testes, but were clearly present after 3 days of culture. Also, these receptors were increased by GnRH treatment and were negatively modulated by gonadotropin [15]. These *in vitro* effects are consistent with the *in vivo* regulation of gonadal GnRH receptors, since GnRH and its agonist analogs increase GnRH receptors in testicular interstitial tissue [3] and pituitary gonadotropin reduced the testicular content of GnRH receptors [23]; also, pituitary gonadotropin reduced, while hypophysectomy increased, the testicular content of GnRH receptors [24].

Recently, inhibitory effects of a GnRH agonist were observed in cultured human granulosa cells [25] despite the reported lack of GnRH receptors in human ovaries [16]. This situation in the ovary may be analogous to the present finding in the fetal testis [15, 22]. It is likely that the number of exposed GnRH receptors in the fresh gonadal tissues (human ovary or fetal testis) is very small and below the detection limits of the present assays, but that additional receptors are unmasked and synthesized during culture. Such increased levels of GnRH receptors may be particularly evident when the fetal testis is released from the effects of endogenous gonadotropins, including both pituitary LH and the gonadotropin-like activity of the rat placenta and circulation [26, 27]. Similarly, the lack of detection of GnRH receptors in the human testis could be due to the negative regu-

Table 1. [^{125}I]GnRH binding to the adult rat testis

Authors	Preparation	K_a $10^9\ M^{-1}$	sites/cell	fmol/10^6 cells	fmol/testis
Clayton *et al.*[3]	27,000 *g* pellet from teased testes	4.0	—	—	—
Perrin *et al.*[6]	Membranes from interstitial cells	3.84	—	—	—
Bourne *et al.*[7]	Membranes from interstitial cells	7.0	—	—	—
Lefebvre *et al.*[8]	Interstitial cells	8.3	2500	—	—
Sharpe *et al.*[9]	Interstitial cells	1.2	—	—	—
Hunter *et al.*[13]	Percoll-purified Leydig cells	—	—	3.8 ± 0.7	—
Aquilano and Dufau[20]	Elutriation-purified Leydig cells	2.42 ± 0.19	3000 ± 400	5.0 ± 0.5	100 ± 10

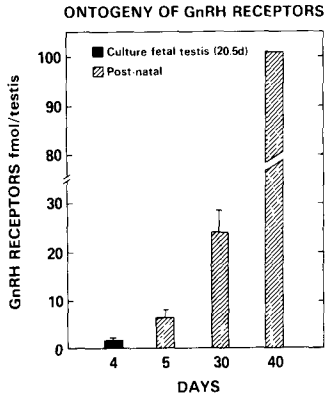

Fig. 2. Ontogeny of GnRH (gonadotropin-releasing hormone) receptors. Homogenates were prepared from testicular interstitial cell fractions of fetal and neonatal Sprague–Dawley rats, and incubated with increasing concentrations of [^{125}I]GnRH-A for 80 min. Note the gradual increase of testicular GnRH receptors binding capacity with age.

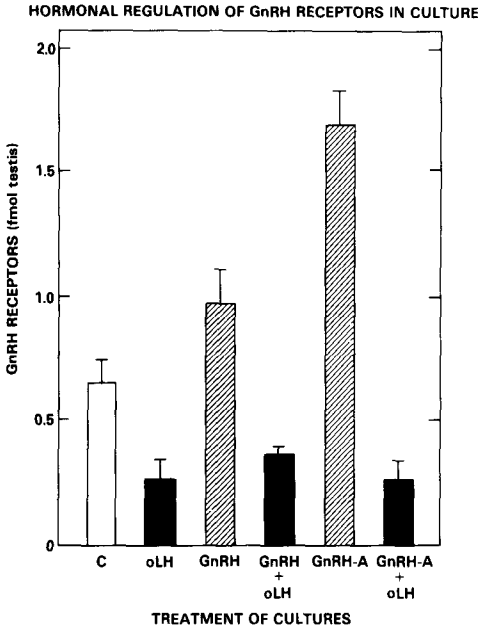

Fig. 3. Hormonal regulation of GnRH receptors in culture. GnRH receptors in 4-day testis cultures [15] incubated in presence and absence of oLH, ovine luteinizing hormone, (1 μg) and GnRH agonist (1 μg). Each assay was performed in triplicate.

lation by the high circulating levels of bioactive LH in man [28], where LH pulses in some cases reach the levels observed in post-menopausal women [28] and at the ovulatory peak of the cycle [29]. This contrasts with the very low bioactive LH levels in male rats. Thus, it is conceivable that the GnRH receptors are down-regulated in the human gonads, and are correspondingly difficult to detect by current techniques.

LEYDIG CELL CULTURE FOR STUDIES OF THE ACTION OF HORMONES IN GENERAL AND APPLIED FOR THE STUDY OF GnRH ACTION

The fetal Leydig cells population can be maintained in culture for at least 78 days with retention of their LH-mediated steroidogenic responses and with expression of functional GnRH receptors [22]. Both steroidogenic activity and receptor-mediated activation of the Leydig cell (i.e. acute testosterone responses to gonadotropin stimulation) are maintained. The gonadotropic responsiveness of fetal cultures was maintained by treatment with ovine LH, as shown in Figs 4 and 5. The trophic hormone was added to the cultures in sufficient doses to maintain receptor-mediated steroidogenic responses, but not so high as to cause induction of the negative regulatory mechanisms observed in adult Leydig cells [15, 22], or to cause heterologous down-regulation of GnRH receptors (Fig. 3). Leydig cells cultured in presence of LH showed intense staining with nitro-blue tetrazolium and cells acquired a rounded shape (Fig. 6), both features are indicators of preservation of steroidogenic activity (Figs 4 and 5) and of increased activity of Δ^5-3β-hydroxysteroid–dehydrogenase/Δ^5-isomerase at this time of culture, as shown by the marked increase in formazan deposits in the Leydig cells cultured in the presence of LH when compared with control cultures. The latter was not noted at earlier times, commensurate with the lack of stimulation on the conversion of Δ-^{5} precursors to Δ^4-metabolites during culture of fetal cells for 4 days (Fig. 14).

In Fig. 4 is shown that testosterone production supported by oLH treatment gradually increased from the time of plating (50×10^4 cells/well) reaching maximal at 6 days. These levels were maintained until 21 days of culture when testosterone production declined significantly, the lowest levels being observed when cells became confluent at 27 days. Following passage of the cultures, a significant increase in testosterone production was observed. The testosterone levels supported by LH were significantly elevated above control until day 78 of culture. The production of progesterone and 17α-OH progesterone showed a similar initial increase but was followed by a gradual reduction to reach control levels at the time of confluency, and were not stimulated after cells were replated at the lower density. The basal production of the 5α-androstan 3α-17β-diol was high after plating and was somewhat inhibited by oLH treatment until day 9. Then basal levels declined to barely detectable values and a marked stimulatory effect was observed in cultures treated with oLH at day 12, levels continued to increase gradually reaching maximal levels at day 24 and maintained high when cells became confluent. After passage little or no production of 5α-androstan 3α-17β-diol was observed either in the presence or absence of hormone. Overall the major steroid produced by the fetal Leydig cell population in culture is testosterone, 17α-OH progesterone and progesterone represented only about 10% and 5α-androstan 3α-17β-diol 25% of the total testo-

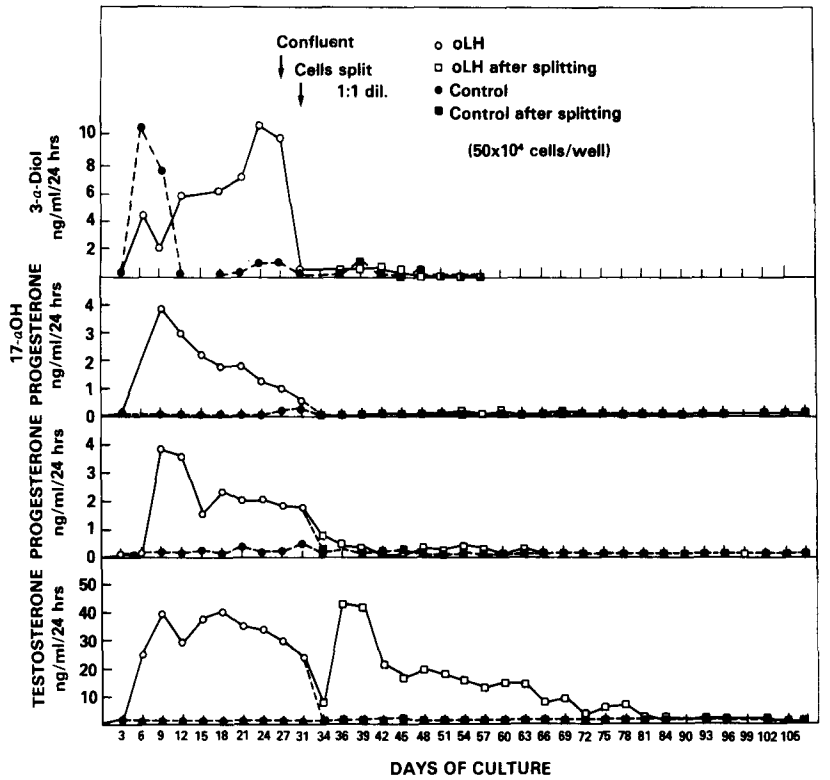

Fig. 4. Steroid production by collagenase dispersed fetal Leydig cells in long-term cultures. Cells treated with 1 μg oLH (every third day added with medium change) continued to produce measurable amounts of steroids for up to 80 days, whereas cells cultured in the absence of oLH showed no steroid production. Cells were plated at a density of 50×10^4 cells/well. 3α-diol = 5α-androstan-3α-17β-diol. Individual points are the mean $\pm$ SE of measurements from 10 cultures.

sterone produced until 30 to 40 days of culture (see also Fig. 5). If cells were plated at a lower density, 12.5×10^4 cells/well the steroid production reached maximal at 9–18 days and these were maintained for all steroids at least until day 36 (Fig. 5). Our results have clearly indicated that confluency is associated with major decrease of testosterone production supported by LH in these cultures.

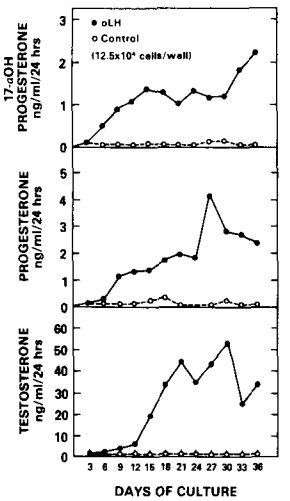

Fig. 5. Steroid production by collagenase dispersed fetal Leydig cells in long-term culture. Cells were treated as in Fig. 4 but plated at lower density (12.5×10^4 cells/well).

Because the inhibitory actions of GnRH are not readily demonstrable during short-term culture of adult Leydig cells, and both LH and GnRH receptors and cytochrome P-450 dependent microsomal enzymes decline to undetectable levels during culture from 12 h to 3 days, [30], cultured fetal Leydig cells (from the late fetal or early postnatal testis) provide a convenient model to examine the direct actions of GnRH in the testis [15, 22]. This system has the advantage that cultures can be used at any time since they maintain GnRH and LH receptor coupling functions and substantial steroidogenic responses that allows accurate dissection of the intermediate steps involved in GnRH action [15, 22], (Figs 4, 5 and 10).

The actions of GnRH on trophic hormone induced steroidogenesis, and also the direct effects of the peptide on basal steroidogenic activity, were examined in cultured fetal Leydig cells. During initial experiments, freshly plated fetal testis cultures were treated with GnRH and the steroidogenic pattern followed for 38 h [15, 22]. GnRH caused a small but significant increase in basal testosterone production ($P < 0.05$), and neither stimulated nor inhibited hormone induced testosterone responses, as illustrated by the similar time-courses of testosterone prod-

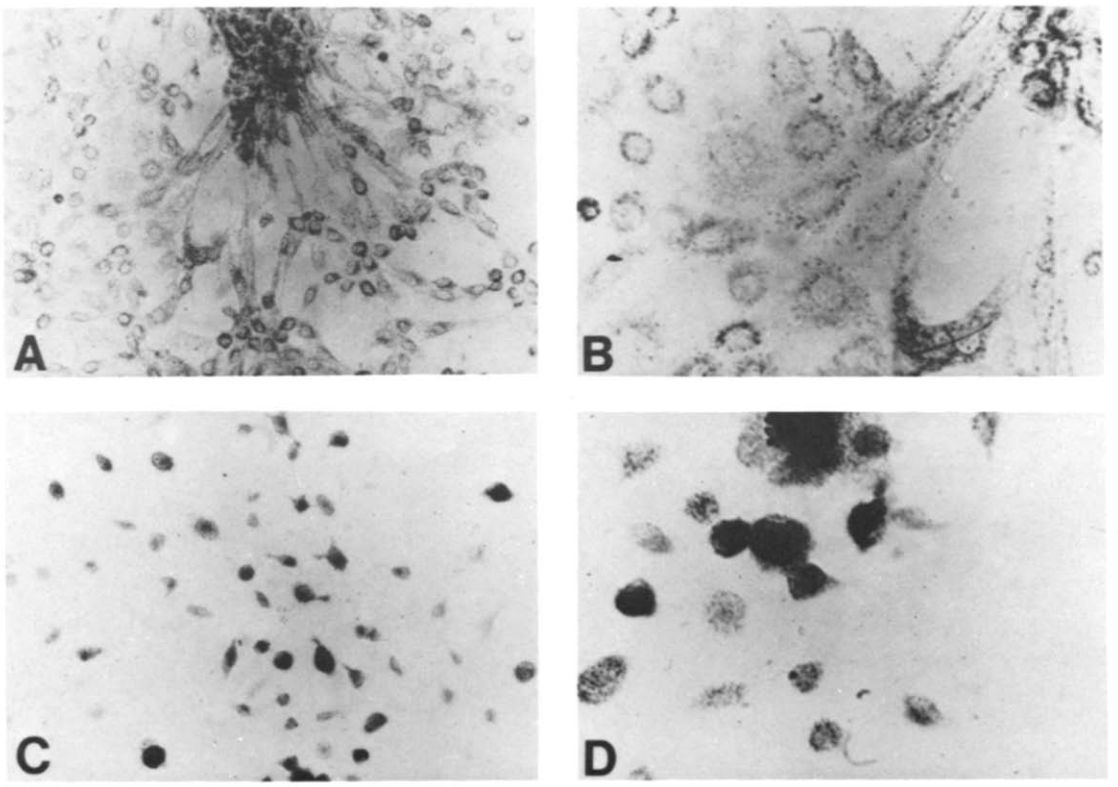

Fig. 6. Histochemical reactions for 3α-hydroxysteroid dehydrogenase activity with nitro blue-tetrazolium [20] in fetal Leydig cell after 7 days of culture A, B,-control C, D-oLH treated. Light micrographs A, C ×16, B, D ×40 magnification.

uction shown in Fig. 7, left. In contrast, when added to cells after 24 h of plating, GnRH markedly reduced the steroid output of cultures treated with LH ($P < 0.001$). This inhibitory effect was evident 6 h after the initiation of treatment, and became more marked after 50 h of GnRH treatment, at 72 h of culture. No effects of GnRH upon basal testosterone production were observed during treatment for 2 days with GnRH, but longer exposure caused a minor inhibition of basal steroid output, which could be attributed to its effect on 17β-hydroxysteroid dehydrogenase (Fig. 7, right and Fig. 8 left, see also Fig. 14). It is not surprising that only minor GnRH effects were observed in freshly established fetal cultures, since GnRH receptors were not detected in gonadal tissue and could only be measured after 2 days in culture [15]. It is likely that the inhibitory effects on the oLH supported steroid production observed after 6 h exposure to GnRH, at 30 h of culture, (Fig. 7, right) were the result of peptide interaction with a small number of receptors that are undetectable by conventional binding assays. When the receptors became readily detectable at 3–4 days of culture, the inhibitory action of GnRH on LH-induced steroidogenesis was markedly enhanced [15, 22] (Fig. 8). These results contrasted with earlier reports on cultured adult Leydig cells, in which a significant stimulation of testosterone production was induced by GnRH after 3 h of culture with no rise in cAMP [12, 13] (LH stimulatory actions caused cAMP increases with 1–5 min [31]). The cited GnRH stimulatory action on the adult Leydig cell was associated with increased phospholipid turnover and does not appear to be mediated by prostaglandins [32, 33]. The GnRH agonist stimulated steroidogenesis is inhibited as was the case for LH by lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA), 3-amino-1[3(trifluoromethylphenyl]-2-pyrazole hydrochloride (BW 755C) and opren R,2-[4-dicloropophenyl]-methyl-5-benzoxazole acetic acid (benoxaprofen), [34]. Although these findings could indicate that a lipoxygenase derivative of arachidonic acid may be important in GnRH action in the Leydig cells as has been proposed in the pituitary, [35] the cited effect of the inhibitors preventing also LH stimulation could be due to nonspecific effects in Leydig cell metabolic processes. On the other hand, recent studies have suggested that arachidonic acid may be important in mediating the direct stimulatory effects of GnRH on Leydig steroidogenesis while its metabolism would not be required for this action [33].

The adult cells in freshly plated cultures, in contrast to the fetal Leydig cells possess a full complement of GnRH receptors and this could account for the observed differences cited above. However, it is important to note that GnRH caused only minor stimulatory effects in the fetal Leydig cells even after the receptors became detectable and/or were up-

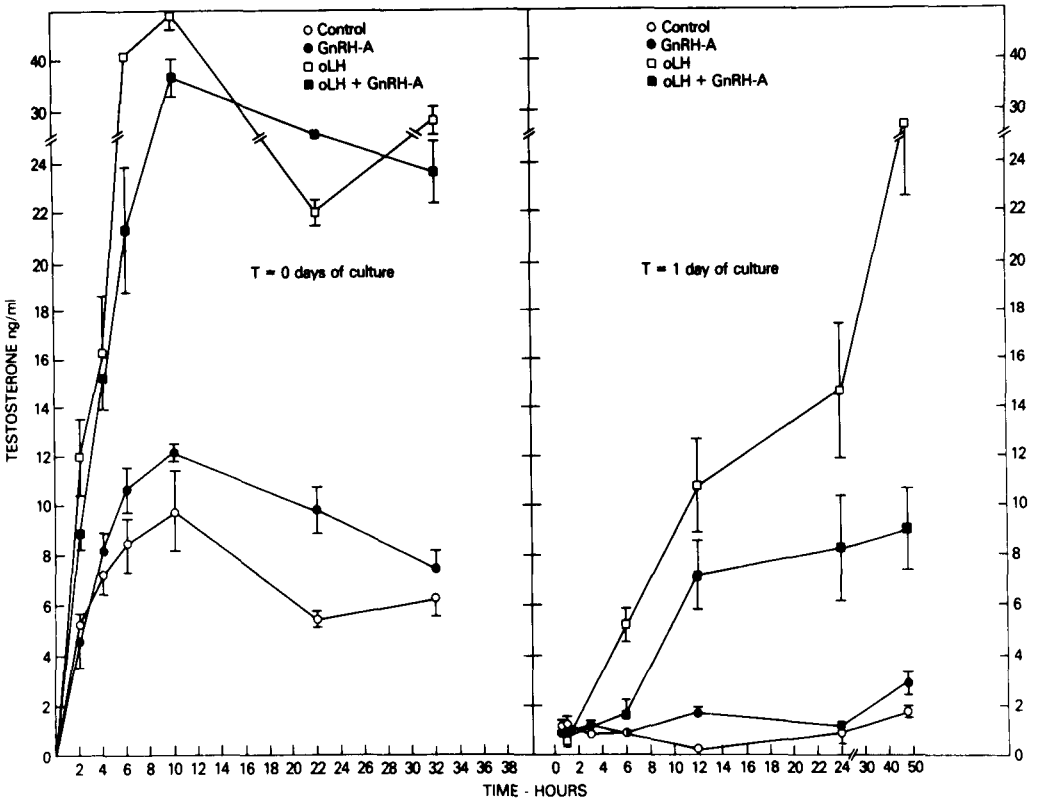

Fig. 7. The time course of GnRH action in the testis. The peptide agonist and or LH were added to the cell culture immediately after plating (left) or 24 h after (right). After 24 h of culture the media was changed and the GnRH and or LH were added to the cultures.

regulated, and that inhibitory effects of GnRH were predominant in this system. The inhibitory effect of GnRH analogs on steroid production were further monitored in cultured fetal testes treated with oLH. As shown in Fig. 8, both native GnRH and the potent agonist significantly reduced basal testosterone levels in oLH-treated cultures at 4 and 7 days ($P < 0.01$). Basal testosterone levels in control cultures did not differ from those of GnRH-treated cultures, but were significantly reduced in GnRH-A treated cultures ($p < 0.01$). As described above, testosterone production was markedly stimulated when oLH-treated cultures were acutely stimulated with hCG (Fig. 8, right), and both GnRH and GnRH-A completely inhibited this gonadotropic stimulation. In contrast, basal testosterone production (in the absence of hCG) was significantly stimulated when compared to cultures not treated with ovine LH ($P < 0.01$) and was not significantly affected by GnRH treatment. No stimulation of testosterone production by hCG was observed in control cultures (not treated with oLH) or in cultures treated with GnRH or GnRH-A alone (Fig. 8, right). Basal progesterone and 17α-hydroxyprogesterone levels (Fig. 8) were significantly elevated ($P < 0.01$) at days 4 and 7 in oLH-treated cultures, regardless of the presence of GnRH or its agonist analog. However, no further increases were observed during hCG stimulation. During a 3-h incubation of 7-day cultures, some increases in basal and hCG-stimulated progesterone and 17α-hydroxyprogesterone production were observed in cultures treated with GnRH and GnRH-A alone (Fig. 8, right upper). Despite the marked reduction of GnRH receptors in oLH-treated cultures, the peptide had an inhibitory effect on testosterone production induced by ovine LH treatment as well as by acute stimulation with hCG [15]. The inhibition of testosterone production was not accompanied by changes in immediate precursors (i.e. 17α-hydroxyprogesterone or progesterone). The fact that these intermediates were not reduced in parallel with testosterone could indicate a lesion of distal enzymes of the androgen biosynthetic pathway, since an exclusive early defect or a combination of both early and late lesions would also cause reduction of 17α-hydroxyprogesterone and progesterone. The lack of the expected compensatory increase of the intermediates indicates that in addition to a late lesion (17α-hydroxylase and/or 17-20 desmolase), an increase in a reductive pathway could be operating and thus preventing the accumulation of progesterone. Since no accumulation of 20α-dihydro-

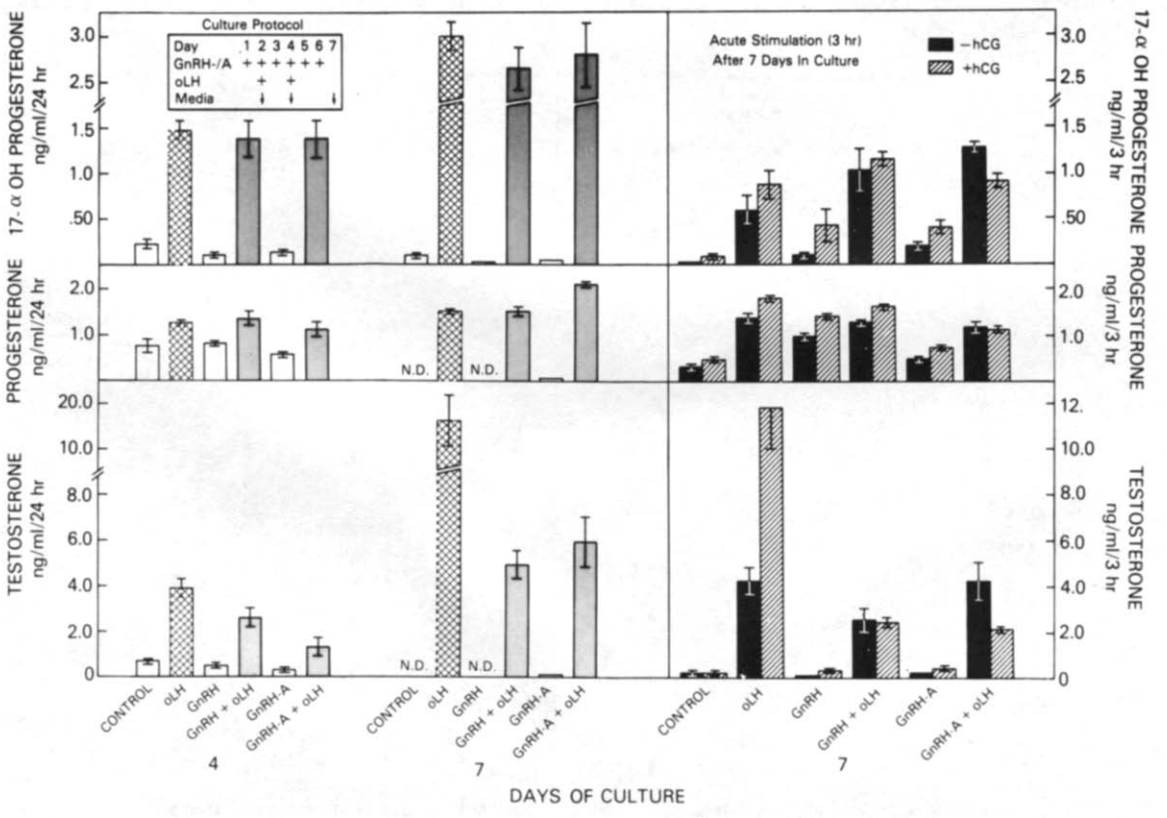

Fig. 8. *In vitro* effects of GnRH and GnRH-A on LH supported basal accumulation (left) and hCG acutely stimulated (right) steroid production (3 h) by fetal testes cultured in presence or absence of oLH 1 μg. Culture protocol is shown in the upper left panel. Media changes are indicated by arrows. Individual points represent the mean ± SE of measurement from 3 cultures.

progesterone was found (see below) other metabolites of the reductive pathways need to be further investigated.

In cultures exposed to a low range of concentrations of GnRH-A, a dose-dependent inhibition of testosterone production was observed from 10^{-11} to 5×10^{-9} M, with half-maximal inhibition at about 3×10^{-10} M (Fig. 9). The concentration of GnRH-A at which inhibitory effects were manifest is commensurate with the affinity of GnRH for its Leydig cell receptors, indicating that functional, high affinity GnRH receptors are responsible for the modulation of Leydig cell responses by GnRH agonists. The inhibitory effect of GnRH on LH supported testosterone production was observed in Leydig cells cultured for 30 days prior to receiving GnRH treatment (Fig. 10).

The functional preservation of mitochondrial steroidogensesis during GnRH action was demonstrated by examining testosterone and pregnenolone production after exposure of cultures to daily additions of oLH, 8 Bromo-cAMP, forskolin, or cholera toxin. In cultures maximally stimulated by LH and cAMP-elevating agents, addition of GnRH-A caused marked (up to 80%) inhibition of testosterone production (Figs 11 and 12). However, pregnenolone accumulation increased in LH-treated cultures (measured in the presence of cyanoketone and spironolactone), was not reduced by GnRH-A (Fig. 13). Also, no effects of GnRH was observed in basal pregnenolone production. The lack of reduction of pregnenolone formation indicates that a late biosynthetic lesion (distal to pregnenolone) is largely responsible for the decrease in androgen production. Furthermore, these studies demonstrated that a major component of the steroid inhibitory effect of GnRH-A occurs beyond cAMP production (Fig. 14). This point was investigated by the addition of radioinert steroids (10^{-5} M) to cultures maintained under

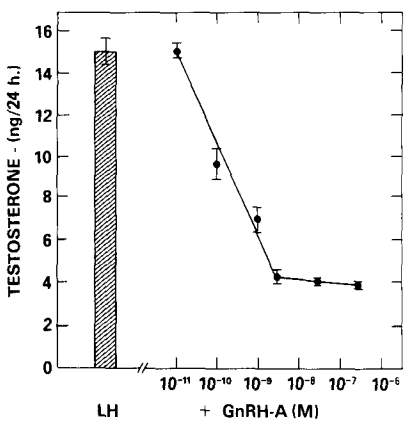

Fig. 9. Dose-dependent inhibition of fetal Leydig cell testosterone production by GnRHA. Cultures were incubated for 4 days with additions of 1 μg oLH and increasing GnRH-A concentrations from 10^{-11} to 10^{-8} M. Points represent the mean $\pm$ SE of data from four cultures.

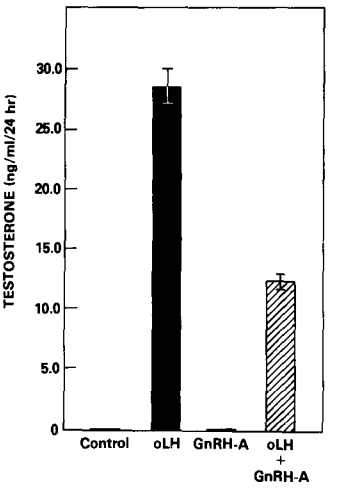

Fig. 10. Effects of GnRH on long-term cultured fetal Leydig cells, prepared by collagenase dispersion of fetal testis (21 day of gestation) and purified by centrifugal elutriation. Cells were maintained in culture with no addition or in presence of 1 μg oLH with media changed every third day. On day 30, cultures were washed and replaced with Medium 199/0.1% Bovine serum albumin/gentamicin (50 μg) for 24 h. Appropriate wells received 1 μg GnRH-A on days 1, 2 and 3. On day 2, media was changed and 1 μg of oLH was added to the appropriate wells along with other appropriate additions (GnRH-A or vehicle). On day 4, media was harvested and testosterone production measured by radioimmunoassay. Individual points represents the mean $\pm$ SE of measurements from 3 cultures. Identical results were obtained with cultures from 1-day old neonatal rats.

control conditions (no further additions) or with additions of oLH or GnRH-A alone and LH + GnRHA and their metabolism quantitated by measurement of the products by specific radioimmunoassays [30]. Addition of pregnenolone to cultures showed no significant changes in progesterone, and when 17α-hydroxypregnenolone was added as substrate no changes were observed in 17α-hydroxyprogesterone with all different incubation conditions showing identical production. The fact that the steroid production was not different in LH treated cells from control cultures (no addition) indicated that Δ^5-3β-hydroxysteroid dehydrogenase–isomerase does not require trophic hormone support under the conditions of these cultures, (See Fig. 14 for cultures conditions). Maintenance of culture for longer periods (7 days or more) required trophic hormone stimulus for preservation of optimal enzymatic activity (Fig. 4). Also, in sort term cultures, the trophic hormone did not stimulate 17β-hydroxysteroid dehydrogenase as shown by the identical production of testosterone in control and LH treated cultures when androstenedione was added as a substrate (Fig. 14, lower panel). In contrast, the conversion from endogenous (metabolic products of pregnenolone when this steroid was used as substrate) or exogenous substrate (progesterone and 17α-hydroxypregnenolone) to 17α-hydroxyprogesterone and testosterone respectively, is highly dependent on the trophic hormone stimulus, as shown

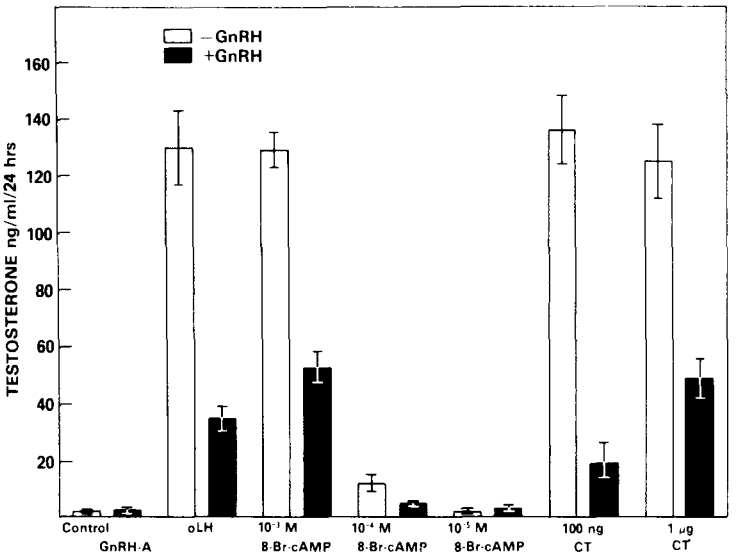

Fig. 11. Leydig cells were cultured with no additions or in presence of 8-Bromo cAMP (10^{-5}–10^{-3} M) choleragen (100 ng and 1 μg) and oLH. Cultures maximally stimulated by oLH, 8-bromo cAMP (10^{-3} M) and choleragen or submaximally by 8-Bromo-cAMP (10^{-4}, 10^{-5} M), showed a marked decrease in testosterone production when cultured in presence of GnRH-A. Fetal cells (21 day) were plated and after 24 h treated daily with GnRH and cyclic AMP elevating agents, ovine LH was added after 48 h with media change. On day 4 media was harvested and steroid production was measured by radioimmunoassay.

by the marked stimulation of 17α-hydroxyprogesterone and testosterone in the LH treated cultures. Significantly higher concentrations of 17α-hydroxyprogesterone were formed from 17α-hydroxypregnenolone than from progesterone added (253 ng/ml/24 h vs 159 ng/24 h). The differences in the conversion to 17α-hydroxyprogesterone from the various substrates added to ovine LH treated cultures, being higher with substrate 17α-hydroxypregnenolone, would indicate that in these cultures the pregnenolone pathway would be active. The latter could also explain the relatively lower testosterone production observed in the LH stimulated cultures when 17α-hydroxyprogesterone was used as substrate when compared with cultures where pregnenolone and 17α-hydroxypregnenolone were added. Furthermore one cannot exclude that the noted differences on the enzymic activities observed (Δ^5 vs Δ^4 pathway) could be due to modulation of microsomal enzyme activities by the substrates added. Our results

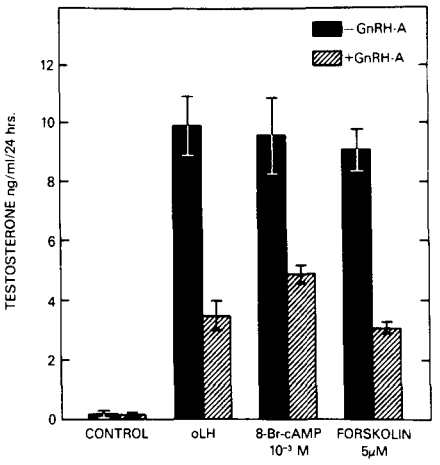

Fig. 12. Leydig cells were cultured with no additions or in the presence of 8-Bromo cAMP (10^{-3} M) and forskolin (5 μM) in presence or absence of GnRHA. Testosterone production supported by these treatments was significantly reduced by GnRH-A (1 μg). Mean ± SE of 3 cultures.

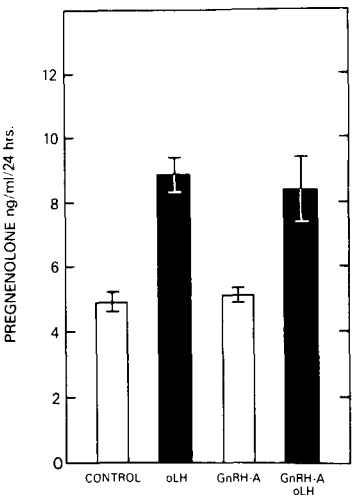

Fig. 13. Pregnenolone production by Leydig cell cultures with daily additions of cyanoketone (10^{-6} M) and spironolactone (10^{-5} M) to inhibit pregnenolone metabolism [22]. Cultures treated with GnRH-A (1 μg) for 3 days were in presence or absence of oLH (1 μg) added with media change on day 2 after initiation of GnRH treatment. On day 4 media was harvested and steroid production was measured by radioimmunoassay. Culture protocol is as Fig. 12.

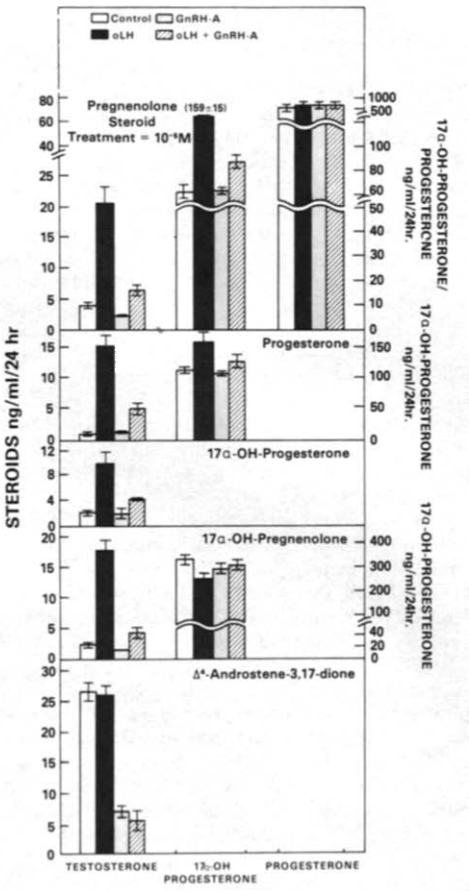

Fig. 14. The effects of LH and of GnRH on the metabolism of pregnenolone progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone and androstenedione by fetal Leydig cells (21-days old) in culture. Cultures were treated with GnRH-A (1 μg) 24 h after plating for 3 days with daily additions of substrates (10^{-5} M) in 20 μl aliquots. Twenty-four cultures for each individual substrate, 12 in presence and 12 in absence of GnRH-A, which received vehicle alone. oLH (1 μg) was added to the cultures with media change at 48 h, along with addition of substrates and GnRH or vehicle in the appropriate wells. Each culture condition is the mean ± SE of $n = 6$ cultures.

clearly indicated that the supportive effects of LH on the production of testosterone shown in cultures incubated with pregnenolone, progesterone, 17α-hydroxypregnenolone are due to increased synthesis and/or activation of 17α-hydroxylase/17-20 desmolase.

Following definition of the effects of trophic hormone, we can conclusively demonstrate that GnRH did not affect Δ^4-3β steroid dehydrogenase since no difference on the production of progesterone from pregnenolone or 17α-hydroxyprogesterone from 17α-hydroxypregnenolone among the different incubations were observed. Also no changes in 20α-dihydroprogesterone (4-pregnen-20α-ol-3-one) were observed with GnRH-A treatment of control cultures (no additions) or in cultures with added substrates pregnenolone and progesterone (not shown). However marked reduction on the oLH supported formation of 17α-hydroprogesterone production was clearly present, when pregnenolone was added as substrate (through endogenously generated intermediate via Δ^4–Δ^5 pathway or both) or when exogenous progesterone was added as subject (direct conversion). These studies have demonstrated that GnRH affects 17α-hydroxylase. Furthermore, since addition of 17α-hydroxyprogesterone or 17α-hydroxypregnenolone along with GnRH showed a decrease in LH supported testosterone production, we conclude that GnRH also affects 17-20 desmolase.

By this study it is clearly observed that GnRH mainly inhibited LH supported production of 17α-hydroxyprogesterone and testosterone (i.e. 17α-hydroxylase, 17-20 desmolase) with minor (Figs 7, 8) or no effects on the basal steroid production (Fig. 14). Finally, GnRH also has a marked inhibitory effect in 17β-hydroxysteroid dehydrogenase as shown by the marked inhibition on the conversion of androstenedione to testosterone.

The steroidogenic lesions observed during GnRH action also shared some common features with those demonstrated during trophic hormone desensitization through the nuclear actions of estradiol [36].

The lack of inhibition of the early activation pathway contrasts with the situation in the ovary, where GnRH inhibits FSH-stimulated progesterone production by decreasing pregnenolone production and 3β-hydroxysteroid dehydrogenase activity as well as by stimulating 20β-hydroxysteroid dehydrogenase [37, 38]. Thus, the major effects in the ovary are probably due to decreased pregnenolone production coupled with increased progesterone metabolism. In the ovary, as in the testis, GnRH blocked the stimulatory actions of exogenous cAMP on steroidogenesis. In addition, GnRH inhibits ovarian GnRH receptor induction and granulosa cell differentiation. GnRH-A has also been shown to inhibit FSH-stimulated adenylate cyclase in the ovary [39]. Although GnRH-A also increased phosphosdiesterase activity in the rat ovary, an action that probably contributes to the inhibitory effect of GnRH-A on FSH-stimulated cAMP production [40], post-cAMP effects related to inhibition of the synthesis and/or activation of type II protein kinase [41] appear to be responsible for inhibitory effect of the peptide on steroidogenesis. Although in the ovary it has been possible to demonstrate a clearcut effect of GnRH in the differentiation of granulosa cells [42], in the testis, in early fetal life, this area has not been investigated mainly due to the lack of suitable *in vitro* systems. It is likely that maternal GnRH contained in the milk as well as the low endogenous LH levels (near undetectable by conventional radioimmunoassay and bioassay) could be responsible for the quiescent state of the postnatal fetal Leydig cell population. This is in marked contrast with the highly active state of the Leydig cell during late fetal life. Also GnRH could exert modulatory actions on the

transition from the fetal Leydig cell population to the adult population at 15–30 days of age [43].

We have demonstrated that the fetal Leydig cell can be maintained in long-term culture with preservation of receptor-activated steroidogenic responses, providing a model system for studies of hormone action in the testis. This system is of particular value for developmental studies and has already proven useful to follow the development of an estrogen induced steroidogenic lesion that is a feature of adult Leydig cells [44]. It is likely that future research with this system will help to clarify the modulatory mechanisms responsible for emergence of the adult Leydig cell population.

REFERENCES

1. Hsueh A. J. W. and Erickson G. F.: Extrapituitary inhibition of testicular function by luteinizing hormone releasing hormone. *Nature* **281** (1979) 66–67.
2. Bambino T. H., Schreiber J. R. and Hsueh A. J. W.: Gonadotropin-releasing hormone and its agonist inhibit testicular luteinizing hormone receptor and steroidogenesis in immature and adult hynophysectomized rats. *Endocrinology* **107** (1980) 908–917.
3. Clayton R. N., Katikinemi M., Chan V., Dufau M. L. and Catt K. J.: Direct inhibition of testicular function by gonadotropin-releasing hormone mediation by specific gonadotropin releasing hormone receptors in interstitial cells. *Proc. natn. Acad. Sci. U.S.A.* **77** (1980) 4459–4463.
4. Clayton R. N. and Catt K. J.: Gonadotropin-releasing hormone receptors: Characterization, physiological regulation, and relationship to reproduction function. *Endocr. Rev.* **2** (1981) 186–220.
5. Hsueh A. J. W.: Direct effects of gonadotropin releasing hormone on testicular Leydig cell. *Ann. N.Y. Acad. Sci.* **383** (1982) 249–271.
6. Perrin M. H., Vaughan J. M., Rivier J. E. and Vale W. W.: High affinity GnRH binding to testicular membrane homogenates. *Life Sci.* **26** (1980) 2251–2256.
7. Bourne G. H., Regiani S., Payne A. H. and Marshall J. C.: Testicular GnRH receptor-characterization and localization in interstitial tissue. *J. clin. Endocr. Metab.* **51** (1980) 407–409.
8. Lefebvre F. A., Reeves J. J., Seguin C., Massicotte J. and Labrie F.: Specific binding of a potent-LHRH agonist in the rat testis. *Molec. Cell. Endocr.* **20** (1980) 127–124.
9. Sharpe R. M. and Frazer H. N.: Leydig cell receptors for luteinizing hormone releasing hormone and its agonists and their modulation by administration or deprivation of the releasing hormone. *Biochem. biophys. Res. Commun.* **95** (1980) 256–262.
10. Dufau M. L., Cigorraga S., Baukal A. J., Sorrel S., Bator J. M., Neubauer J. F. and Catt K. J.: Androgen biosynthesis in Leydig cells after testicular desensitization by luteinizing hormone-releasing hormone and human chorionic gonadotropin. *Endocrinology* **10** (1979) 1314–1321.
11. Catt K. J., Harwood J. P., Clayton R. N., Davies T. F., Chan V., Katikineni M., Nozu K. and Dufau M. L.: Regulation of peptide hormone receptors and gonadal steroidogenesis. *Recent Prog. Horm. Res.* **36** (1980) 557–662.
12. Sharpe R. M. and Cooper I.: Stimulatory effect of LHRH and its agonists in Leydig cell steroidogenesis *in vitro*. *Molec. Cell. Endocr.* **26** (1982) 141–150.
13. Hunter M. G., Sullivan M. H. F., Dix C. J., Aldred L. F. and Cooke B. A.: Stimulation and inhibition by LHRH analogues of cultured rat Leydig cell function and lack of effect on mouse Leydig cells. *Molec. Cell. Endocr.* **27** (1982) 31–44.
14. Hsueh A. J., Bambino T. H., Liu-Zhi Zihuang, Welsh T. H. and Ling C. N.: Mechanism of the direct action of gonadotropin-releasing hormone and its antagonist on androgen biosynthesis by cultured rat testicular cells. *Endocrinology* **112** (1983) 1653–1661.
15. Dufau M. L., Warren D. W., Knox G. F., Loumaye E., Castellon M. L., Luna S. and Catt K. J.: Receptors and inhibitory actions of gonadotropin-releasing hormone in the fetal Leydig cell. *J. biol. Chem.* **259** (1984) 2896–2899.
16. Clayton R. N., Huhtaniemi I. T.: Absence of gonadotropin-releasing hormone receptors in human gonadal tissue. *Nature* **299** (1982) 5878.
17. Sharpe R. M., Fraser H. M., Cooper I. and Rommerts F. F. G.: The secretion measurement and function of a testicular LHRH like factor. *Ann. N.Y. Acad. Sci.* **383** (1982) 272–292.
18. Sharpe R. M., Frazer H. M., Cooper I. and Rommerts F. F. G.: Sertoli–Leydig cell communication via an LHRH-like factor. *Nature* **290** (1981) 785–787.
19. Hedger M. P., Robertson D. M., Browne C. A. and de Kretser M.: Studies on the identification of LHRH-like peptides in the rat testis. *Ann. N.Y. Acad Sci.* **438** (1984) 371–381.
20. Aquilano D. and Dufau M. L.: Functional and morphological studies on isolated Leydig cells. Purification by centrifugal elutration and metrizamide gradients. *Endocrinology* **114** (1984) 499–510.
21. Aquilano D. and Dufau M. L.: Studies on Leydig cell purification. *Ann. N.Y. Acad. Sci.* **438** (1984) 237–258.
22. Knox G. F., Castellon M. L., Catt K. J. and Dufau M. L.: Fetal Leydig cell culture–an *in vitro* system for the study of GnRH action. *7th Int. Congr. of Endocr.*, Quebec City, Abstract No. 1461 (1984) Excerpta Medica, Amsterdam.
23. Sharpe R. M., Fraser H. M., Cooper I. and Rommerts F. F. G.: The secretion measurement and function of a testicular LHRH like factor. *Ann. N.Y. Acad. Sci.* **383** (1982) 272–294.
24. Bownie G. A. and Marshall J. C.: Anterior pituitary hormone regulation of testicular gonadotropin-releasing hormone receptors. *Endocrinology* **115** (1984) 723–727.
25. Tureck R. W., Mastroianni L., Blasco L. and Strauss J. F.: Inhibition of human granulosa cell progesterone secretion by a gonadotropin-releasing hormone agonist. *J. clin. Endocr. Metab.* **54** (1982) 1078–1080.
26. Blank M. S., Dufau M. L. and Friesen H. G.: Demonstration of potent gonadotropin-like biological activity in the serum of rats during mid-pregnancy. *Life Sci.* **25** (1979) 1023–1028.
27. Blank M. S. and Dufau M. L.: Rat chorionic gonadotropin: Augmentation of bioactivity in the absence of the pituitary. *Endocrinology* **112** (1983) 2200–2201.
28. Dufau M. L., Veldhuis J. D., Fraioli F., Johnson M. L. and Beitins I. Z. Mode of secretion of bioactive luteinizing hormone in man. *J. clin. Endocr. Metab.* **57** (1983) 993–1000.
29. Dufau M. L., Pock R., Neubauer A. and Catt K. J.: *In vitro* bioassay of LH in human serum: The rat interstitial cell testosterone (RICT) assay. *J. clin. Endocr. Metab.* **42** (1976) 958–969.
30. Dufau M. L., Cigorraga S. B., Baukal A. J., Bator J. M., Sorrell S. H., Neubauer J. F. and Catt K. J.: Steroid biosynthetic lesions in gonadotropin-desensitized Leydig cells. *J. steroid Biochem.* **11** (1979) 193–198.
31. Dufau M. L. and Catt K. J.: Gonadotropin receptors and regulation of steroidogenesis in testis and ovary. In

Vitamins and Hormones (Edited by P. Munson and E. Diczfaluzy). Academic Press, New York, Vol. 36 (1978) 461–600.
32. Molcho J., Zakut H. and Naor Z.: Gonadotropin-releasing hormone stimulates phosphatidylinositol labeling and prostaglandin production in Leydig cells. *Endocrinology* **114** (1984) 1048–1050.
33. Cooke B. A., Dix C. J., Habberfield A. D. and Sullivan M. H. F.: Control of steroidogenesis in Leydig cells: Role of Ca^{2+} and lypoxygenase products in LH and LHRH agonist action. *Ann. N.Y. Acad. Sci.* **438** (1984) 269–282.
34. Lin T.: Mechanism of action of gonadotropin-releasing hormone stimulated Leydig cell steroidogenesis. The role of arachidonic acid and Ca/phospholipid dependent protein kinase. *Life Sci.* **36** (1985) 1255–1264.
35. Catt K. J., Loumaye E., Wynn C., Suarez-Quain Kiesel L., Iwashita M., Hirota K., Morgan R. and Chang J.: Receptor-mediated activation mechanisms in the hypothalamic control of pituitary gonadal function. In *Endocrinology* (Edited by F. Labrie and L. Proulx). Elsevier (1984) pp. 57–65.
36. Dufau M. L., Winters C. A., Hattori M., Aquilano D., Baranao J. L. S., Nozu K., Baukal A. and Catt K. J.: Hormonal regulation of androgen production by the Leydig cell. *J. steroid Biochem.* **20** (1984) 161–173.
37. Jones P. B. C. and Hsueh A. J. W.: Direct stimulation of ovarian progesterone metabolizing enzyme by gonadotropin-releasing hormone in cultured granulosa cells. *J. biol. Chem.* **256** (1981) 1248–1254.
38. Jones P. B. C. and Hsueh A. J. W.: Regulation of ovarian 3β-hydroxysteroid dehydrogenase by GnRH and follicle-stimulating hormone in cultured rat granulosa cells. *Endocrinology* **110** (1982) 1663–1671.
39. Knecht M., Ranta T., Katz M. S. and Catt K. J.: Regulation of adenylate cyclase activity follicle stimulating hormone and a gonadotropin releasing hormone agonist in culture rate granulosa cells. *Endocrinology* **112** (1983) 1247–1255.
40. Knecht M., Ranta T. and Catt K. J.: Hormonal regulation of a plasma membrane phosphodiesterase in differentiating granulosa cells. Reciprocal actions of follicle-stimulating hormone and in gonadotropin releasing hormone agonist on cAMP degradation. *J. biol. Chem.* **258** (1983) 12420–12426.
41. Darbon J. M., Knecht M., Ranta T., Dufau M. L. and Catt K. J.: Hormonal regulation of cyclic AMP-dependent protein kinase in cultured ovarian granulosa cells. Effects of follicle-stimulating hormone and gonadotropin-releasing hormone. *J. biol. Chem.* **259** (1984) 14778–14782.
42. Ranta T., Knecht M., Baukal A. J., Korhonen M. and Catt K. J.: GnRH agonist-induced inhibitory and stimulatory effects during ovarian follicular maturation. *Molec. Cell. Endocr.* **35** (1984) 55–63.
43. Smith S. S. and Ojeda S. R.: Maternal modulation of infertile ovarian development and available ovarian luteinizing hormone-releasing hormone (LHRH) receptors via milk LHRH. *Endocrinology* **115** (1984) 1973–1983.
44. Tsai-Morris C. H., Knox G., Luna S., Ciocca D. and Dufau M. L.: Acquisition of estradiol-mediated regulatory mechanism of steroidogenesis in cultured fetal rat Leydig cells. *Endocrinology* **116A** (1985) (Abstract No. 70), p. 18.