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GONADOTROPHIN RELEASING HORMONE RECEPTOR REGULATION IN RELATIONSHIP TO GONADOTROPHIN SECRETION

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Summary—The relationship between pituitary GnRH receptors (GnRH-R) and LH responsiveness to GnRH stimulation is not straightforward. In some circumstances, e.g. post-gonadectomy of rats, in lactating rats, during the rat, hamster and monkey oestrous cycles there appears to be a good positive correlation between GnRH-R, basal serum LH values and LH responses to exogenous GnRH. However, in mice following gonadectomy GnRH-R fall by 50% while serum LH levels rise by 10-fold, and in cultured pituitary cells, GnRH exposure increases GnRH-R yet desensitises cellular responsiveness to subsequent GnRH stimulation. Thus, our original hypothesis that GnRH-R regulation was closely coupled to gonadotroph secretory function does not always hold. Further, we and others, using the rat as an experimental model, hypothesised that the pituitary GnRH receptor content reflected the level of previous pituitary exposure to endogenous GnRH. This view is supported with studies in the GnRH deficient hypogonadotrophic hypogonadal (*hpg*) mouse in which exogenous GnRH rapidly normalises GnRH-R from very low levels, and is accompanied by rapid activation of pituitary FSH synthesis. However, the post-castration fall in GnRH-R in mice, which is opposite to that in rats, does not appear to be so closely related to endogenous GnRH secretion and cannot be reversed by exogenous GnRH. Using the ovariectomised mouse as an experimental model, evidence has been obtained that estradiol, in addition to GnRH, is essential for maintenance of pituitary GnRH-R in this species. Exogenous estradiol stimulates GnRH-R in OVX mice while it reduces the high values in OVX rats. In female mice estradiol and GnRH have additive stimulatory effects on GnRH-R. Thus, there is species variability in the predominant hormonal regulation of GnRH receptors. In rat pituitary cells *in vitro* up-regulation of GnRH-R can be effected by several agents which stimulate LH release (GnRH, KCl, DbCAMP) as well as some which do not (Ca inophore at low concentrations). Receptor up-regulation requires Ca^{2+} mobilisation and protein synthesis. The data obtained from several *in vivo* and *in vitro* model systems supports the conclusion that GnRH receptor changes represent another, medium-term, consequence of GnRH action on the gonadotroph and are not always a locus for the modulation of gonadotrophin secretion and synthesis.

INTRODUCTION

It is now well recognised that gonadotrophin-releasing hormone (GnRH), in common with other oligopeptide and glycoprotein hormones, initiates its actions through stereospecific receptors (GnRH-R) on the surface of the gonadotroph [1]. A variety of cellular biochemical events follow GnRH-receptor interaction which culminate in the release of LH. These include: (1) rapid hydrolysis of phosphatidyl inositol and increased phospholipid turnover, (2) calcium influx and/or mobilisation from intracellular stores to increase free intracellular Ca^{2+} with activation of calmodulin dependent enzymes; (3) formation of arachidonic acid metabolites; (4) possible activation of protein kinase C [2]. While some of the events precede gonadotrophin secretion others may be involved in the fusion of the LH secretory granule and plasma membrane to effect exocytosis. Termination of GnRH action occurs by the process of internalisation of the hormone–receptor complex [3, 4] as occurs for other ligands which effect their actions by surface receptors [5]. While immunocytochemical studies [6] have suggested the presence of GnRH receptor complexes within gonadotroph secretory granules in intact cells, these have not been measurable in secretory granule preparations from the pituitary [7, 8]. Indeed, *in vitro* autoradiographic studies have localised intracellular GnRH receptor complexes to lysosomes/multivesicular bodies and Golgi complexes [9, 10]. Thus, the bulk of evidence indicates that free GnRH-R are exclusively located in the plasma membrane.

Initial attempts to measure GnRH-R employed purified bovine pituitary plasma membrane preparations or crude rat pituitary membrane preparations as sources of receptor, and iodinated natural sequence GnRH as the radioligand. This assay system was unsuitable for the accurate quantitative study of GnRH-R changes because the ligand bound predominantly to low affinity sites ($K_d = 10^{-7}$ M), which probably represented interaction of the hormone with

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contaminating peptidases in lysosomal membrane fragments [11]. With the advent of degradation resistant superagonist analogues of GnRH (GnRH-A), we [12] and others [13] could accurately quantitate a single class of high affinity ($K_d = 10^{-10}$ M) binding sites in pituitary membrane preparations [1]. This improved assay could also be applied to GnRH-R measurement in unfractionated homogenates of rodent pituitary glands, thereby allowing correlation between GnRH-R, pituitary, and serum gonadotrophins in individual animals [14, 15]. Subsequently, with minor modifications, the [^{125}I]GnRH-analogue RRA has been used for studies of GnRH receptor regulation in cultured pituitary cells.

This review will, therefore, concentrate on the physiological and biochemical regulation of GnRH receptors in the pituitary since this is the primary physiological target for the hypothalamic decapeptide. High-affinity GnRH-R have been located in rat gonads [16] and low affinity binding sites measured in human placenta and gonads [17, 18]. In the rat these receptors are functionally active since GnRH can exert either inhibitory or stimulatory actions in the ovary and testis [16]. Nevertheless, the precise physiological functions, and the naturally occurring ligand, for these receptors remain to be identified.

GnRH RECEPTORS *IN VIVO*

The GnRH receptor autoregulation concept

With the availability of the reliable GnRH-RRA the initial studies addressed the gross relationship between GnRH-R, serum, and pituitary LH and FSH concentrations in different hormonal environments. These studies were largely confined to the rat and have been reviewed in detail elsewhere, but are summarised here in Table 1.

Having documented GnRH-R changes it was next possible to ask the question: what hormone(s) is (are) responsible for this? A positive correlation between GnRH-R and serum gonadotrophins was apparent in two situations: (1) after orchidectomy or ovariectomy and (2) in the lactating rat, situations which are believed to be associated with increased and decreased endogenous hypothalamic GnRH secretion, respectively. In addition, during prepuberty in the rat GnRH-R are also high, a time when the pituitary is most responsive to GnRH. These apparent associations led to the hypothesis that GnRH, by a direct action on gonadotrophs, was the major hormonal determinant of the pituitary GnRH-R content. If this was correct then the GnRH-R content would provide a qualitative index of prior pituitary exposure to endogenous GnRH.

To test this hypothesis directly we, and others, have determined the effects of elimination of endogenous GnRH secretion on the post-castration GnRH-R and serum gonadotrophin responses in rats. The experimental approaches adopted were: (1) to eliminate the storage of GnRH in the median eminence by either mechanical or electrolytic lesions; (2) to immunoneutralise endogenous GnRH; (3) to block GnRH access to its receptors with a GnRH antagonist. The results of these experiments are summarised in Table 2, and indicate the importance of endogenous GnRH for the post-orchidectomy GnRH-R increase in male rats. Using the same GnRH antiserum, administered once daily for 7 days, commencing at the time of castration, we could also prevent the post-ovariectomy GnRH-R and serum gonadotrophin increase in female rats (Table 3). Furthermore, when these same treatments, particularly the antiserum, were applied to intact male and female animals GnRH-R, as well as serum LH and FSH, were reduced by about 30–60% indicating the importance of pituitary exposure to the hormone for maintaining a "normal" receptor complement (Table 3). These data provide strong indirect evidence for the proposed "autoregulation" hypothesis and indicate that GnRH effects on its own receptor are not secondary to changes in gonadal steroid secretion (*vide infra*).

Further support for the GnRH up-regulation hypothesis is obtained from studies in which exogenous GnRH or GnRH-A administered intermittently, has been shown to increase receptors by 100–200% in both male and female rats [14, 15]. However, it has not been possible to raise the already high post-

Table 1. GnRH receptor changes in various circumstances in the rat

	GnRH-R changes	Serum gonadotrophins
Orchidectomy Ovariectomy	↑ 100–200%	↑ 5–10-fold
Oestrous cycle	↑ 100–200% on dioestrous/proestrous ↓ at time of LH surge	No change until LH surge
Lactation	↓ 50%	↓ 50%
Induced hyperprolacinaemia	Attenuated rise post-castration; reduced in intact animals	Reduced rise postcastration
Sexual maturation	Elevated prepubertally and fall with rising gonadal steroid secretion	As GnRH-R

Table 2. Effect of interruption of endogenous GnRH secretion on GnRH-R and serum gonadotrophin responses to orchidectomy of rats

Experimental design	Result: GnRH-R increase	Result: Increased serum LH	Reference
(1) Median eminence lesion (M.E.L.) M.E.L. + exogenous GnRH	Prevented-GnRH-R below intact control restored	Below intact restored	[15, 56]
(2) GnRH antiserum GnRH-AS + exogenous GnRH-A	Prevented-below intact control partially restored	Prevented Undetectable*	[15, 55]
(3) GnRH antagonist infusion*	Prevented-below intact control	Undetectable	[56]

*Measured 16 h after last GnRH-A injection, therefore not expected to be raised. GnRH-A = D-Ser-(tBu^6)-des-Gly^{10}-GnRH-*N*-ethylamide.
†Continuous infusion for 7 D from intraperitoneal osmotic minipumps.

castration levels of GnRH-R by exogenous GnRH or GnRH-A administration. Indeed, during studies in which either GnRH or its agonist analogue were infused continously from osmotic minipumps to determine the extent of receptor down-regulation in conditions of pituitary desensitisation, we found that infusion doses producing physiological serum GnRH levels (40–100 pg/ml) could cause a small but significant receptor increase (30–40%) [19].

It should be emphasised that none of these changes in receptor content could be accounted for by alteration in the affinity of the receptors, or by interaction of the GnRH antiserum with GnRH binding sites.

Further *in vivo* evidence for a direct up-regulatory action of GnRH on its own receptors is provided by studies in the GnRH deficient, hypogonadotrophic hypogonadal (*hpg*) mouse (v.i.). Much *in vitro* evidence, summarised below, also gives support to the hypothesis.

Thus, it has beome widely accepted that GnRH is one of the few hormones, along with angiotensin (II) and perhaps prolactin [5] which induces its own receptors and that these may be a prerequisite for, or at least part of, an appropriate physiological response to altered hormonal milieu.

GnRH-R REGULATION IN MICE

From the data accumulated in rats it became important to establish whether this direct pituitary receptor up-regulatory action of GnRH was universally applicable, i.e. was the homologous ligand the major determinant of GnRH receptor changes in all species. In an attempt to verify this we turned to mice, using either the normal littermates or the mutant *hpg* (GnRH deficient) mouse (strain C3H/HeH/101H F_1 hybrid intercrosses) or BALB/c white mice.

GnRH deficient (hpg) mice

These animals have a congenital isolated deficiency of hypothalamic GnRH and fail to undergo sexual maturation at puberty, the gonads and accessory sex organs remaining miniscule. These animals arose by spontaneous mutation at the Atomic Energy Research Establishment, Harwell, Oxon, U.K. [20] and are analogous to the uncommon human condition of isolated gonadotrophin deficiency with or without Kallman's syndrome (anosmia and colour blindness). The pituitary content of LH and FSH, though detectable, is very low, and GnRH receptors are 30% of normal littermate values [21]. The low GnRH-R levels are not further reduced by GnRH-AS treatment. Otherwise, all other endocrine function is normal [20]. When exogenous GnRH is administered to *hpg* male mice in physiological pulses every 2 h there is a brisk (< 24 h) doubling of GnRH-R which precedes the major increase in pituitary and serum FSH and LH levels [21]. Furthermore, we have found [22] that a single injection of GnRH is sufficient to double GnRH-R in both male and female *hpg* mice between 8–12 h after the injection, and occurs in the absence of the gonads. This contrasts with the failure of a single injection of GnRH to up-regulate its own receptors in normal male mice [22] and in ovariectomised normal female mice (v.i.). Similar up-regulation of GnRH-R has been

Table 3. Effect of a GnRH antiserum† on pituitary GnRH receptors, serum, and pituitary gonadotrophins in intact and castrated female rats

Groups *n*	Intact 6	Intact + AS 6	OVX 6	OVX + AS 6
GnRH-R (fmol/pit)	149 ± 16	53.4 ± 13.5*	231 ± 29*	48.3 ± 2.7*
Serum LH (ng/ml)	24 ± 6	< 6.5	167 ± 27	< 6.5
Serum FSH (ng/ml)	154 ± 46	64 ± 9*	860 ± 75	228 ± 8
Pit. LH (μg/pit)	258 ± 24	311 ± 21	449 ± 36	348 ± 14
Pit. FSH (μg/pit)	28.2 ± 3	26 ± 2	133 ± 17	73 ± 7*

Values are $\bar{x} \pm$ SE.
*$P < 0.01$ vs intact controls.
†0.25 ml of sheep anti-GnRH serum was administered s.c. only daily for 7 days commencing at the time of ovariectomy. Animals sacrificed 16 h after last injection.

observed in *hpg* mice of both sexes when GnRH is provided by means of a foetal hypothalamic implant into the 3rd ventricle of the mutant recipient. Thus, GnRH-R up-regulation is a well-documented feature of the GnRH deficient animals and parallels the general trophic effects of this hormone on gonadotrophin synthesis and cellular morphology [23]. Indeed, this animal is ideal for the *in vivo* investigation of hormonal regulation of gonadotroph function since all effects must be a consequence of a direct pituitary site of action, and as such may provide an *in vivo* pituitary cell culture system. Thus, the time-course of GnRH-R induction after a single exposure of the *hpg* pituitary to GnRH *in vivo* is almost identical to that observed in pituitary cells *in vitro* (vi.). Moreover, GnRH-R induction *in vivo* is also dependent upon protein synthesis [22]. These latter observations confirm that the biochemical mechanism for receptor up-regulation elucidated from *in vitro* studies are most likely to apply *in vivo*.

Therefore, in the GnRH deficient animal the exogenous decapeptide up-regulates its own receptors, as does both endogenous and exogenous GnRH in rats. But, is endogenous GnRH the *primary* determinant of the pituitary GnRH receptor content in *normal* mice?

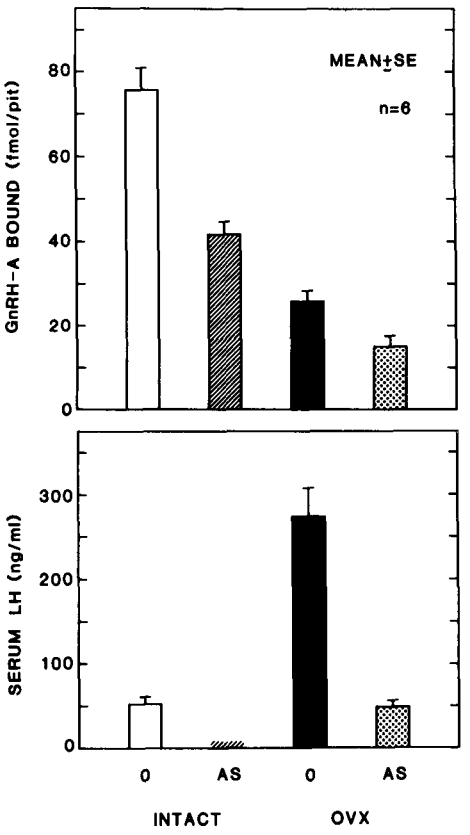

Fig. 1. Effect of a GnRH antiserum (AS), 50 μl s.c. for 7 days, on GnRH receptors (upper panel) and serum LH (lower panel) in intact and ovariectomised (OVX) adult female mice. Antiserum injections commenced at the time of OVX. Methods as described in Naik *et al.*, 1984a and b.

GnRH receptor regulation in normal mice

To address the last question, and to investigate the relationship between GnRH-R and gonadotropin secretion in mice, these parameters have been analysed after orchidectomy, ovariectomy, and during lactation, in mice with intact hypothalamic GnRH secretion. To our surprise we found that, in contrast to rats, GnRH-R fell consistently and persistently (up to 3 months) after gonadectomy by about 50%, despite a 6–10 fold increase in serum LH and FSH concentrations [24, 25]. Furthermore, in the *Tfm* mouse, with inherited androgen resistance, we also observed a similar receptor decrease in the presence of high gonadotrophins. The receptor affinity was unaltered in all instances, and the receptor fall occurred in two strains of mouse examined. Replacement of testosterone in males, and estradiol plus progesterone in females, could prevent the receptor decrease and the gonadotropin increase. In contrast to the discrepancy in post-castration GnRH-receptor responses between rats and mice we found that the lactational changes in mice were identical to those in rats [25]. Thus, after gonadectomy of mice we observed, for the first time, a negative correlation between GnRH-R and serum gonadotrophin levels, which questioned our GnRH autoregulation hypothesis, at least as applied to mice after castration.

Three possible explanations for the post-castration GnRH-R fall were considered: (1) this represented down-regulation or receptor occupancy as a consequence of elevated endogenous GnRH secretion; (2) endogenous GnRH secretion was "relatively" deficient, unlikely in view of the elevated serum gonadotropins; (3) the hypothesis that GnRH was the primary hormonal regulator of its own receptors in the normal mouse pituitary was incorrect.

A number of experiments have been performed to resolve these possibilities and for these purposes we have used female mice ovariectomised at undefined random stages of their oestrous cycles. We reasoned that if receptor occupancy or down-regulation was the mechanism then immunoneutralisation of endogenous GnRH should prevent the post-ovariectomy fall in GnRH-R. When GnRH-AS was administered at the time of ovariectomy the receptor fall was *accentuated* (by 50%) rather than prevented, despite complete suppression of the serum gonadotrophin rise (Fig. 1). This seemed to exclude option 1 (v.s.). Indeed, the further fall in GnRH-R after GnRH antiserum treatment implied a requirement for endogenous GnRH in the maintenance of the reduced GnRH-R complement after castration of normal mice. That GnRH is involved in its own receptor regulation in normal mice is also indicated by a 50% fall in GnRH-R in *intact* female mice treated with the antiserum (Fig. 1).

However, it appeared that the effect of endogenous GnRH on its own pituitary receptors in mice may not be the consequence of a direct action on the gonad-

Table 4. Effect of oestradio-17β (E_2) treatment (300 ng/day) × 7 days in intact normal and hpg female mice

Groups	♀ Intact control	♀ + E_2	hpg	hpg + E_2
GnRH-R (fmol/pit)	11.7 ± 0.3	9.83 ± 0.5	4.1 ± 0.4	7.2 ± 0.7
Serum LH (ng/ml)	80 ± 8	73 ± 9	< 24	37 ± 5
Serum FSH (ng/ml)	205 ± 5	170 ± 10	< 50	120 ± 10
pit LH (μg/pit)	19.1 ± 4.1	15.0 ± 1.8	4.1 ± 0.29	4.3 ± 0.40
pit FSH (μg/pit)	2.86 ± 0.42	2.4 ± 0.42	0.29 ± 0.13	1.4 ± 0.13
Ovarian wt (mg)	11.0 ± 1.3	12.5 ± 1	0.67 ± 0.1	2.5 ± 0.6
Uterine wt (mg)	124.6 ± 12.5	221.5 ± 15	7.17 ± 0.3	47.6 ± 2.8

otroph, since exogenous GnRH was unable to up-regulate GnRH-R in female mice in the absence of the gonads, though the replacement regimen employed was clearly effective in producing desensitisation of LH secretion. Further, the effectiveness of the exogenous GnRH was substantiated by the ability of the hormone to increase GnRH by 30–40% in *intact* female mice, at the same time as causing LH desensitisation. The conclusion from these experiments is that GnRH receptor up-regulation in intact mice is secondary to its stimulation of a gonadal product, probably steroid hormones. Since we had previously shown [25] that the post-OVX GnRH-R fall was largely restored by exogenous E_2 and progesterone (P) this was consistent with the view that the major explanation for the GnRH receptor fall post-OVX was withdrawal of the maintaining effect of $E_2 + P$. Therefore, the fall in GnRH-R in antiserum treated intact mice is largely due to the secondary reduction in gonadal steroidogenesis. Nevertheless, since $E_2 + P$ treatment of OVX female mice did not restore GnRH-R entirely to normal values it remained possible that some synergistic action between steroids and GnRH, acting directly at the pituitary level, is required for maintaining a "normal" receptor complement in female mice.

To obtain definitive evidence for a direct up-regulatory effect of E_2 on GnRH-R *in vivo* the steroid was injected daily for 7 days to gonadally intact GnRH deficient, *hpg*, female mice. The regimen doubled GnRH-R, without affecting serum or pituitary gonadotropins, though values did not reach those of the intact normal females (Table 4). Addition of progesterone to E_2 treatment of *hpg* females for 14 days produced greater up-regulation than E_2 alone, and when exogenous GnRH was also given (60 ng × 5 daily) values reached those of the intact normal female mice. The results of these same treatments were similar in ovariectomised *hpg* female mice indicating that endogenous steroids did not contribute to the receptor changes. These data confirm the view that GnRH, in addition to ovarian steroids, is required for normalisation of GnRH-R and acts at the pituitary level. Thus, in contrast to rats in whom ovarian steroids are not required for GnRH-R maintenance [1], GnRH-R of female mice are clearly dependent on estrogen.

Limited studies to define the mechanism of the post-orchidectomy fall in GnRH-R in male mice show that this is also further accentuated by GnRH antiserum treatment and cannot be reversed by single injections of GnRH, though testosterone replacement can clearly prevent the post-orchidectomy receptor fall and serum gonadotrophin rise [24]. In contrast to gonadally intact female mice, a single injection of GnRH to normal male mice fails to induce GnRH-R [22]. Thus, the GnRH-R maintenance in male mice is also dependent upon gonadal steroids, as in females, but perhaps to a lesser extent.

Many of the studies in normal and *hpg* mice show a dissociation between GnRH-R regulation and serum or pituitary gonadotrophin levels, indicating that these are clearly regulated independently. Thus, the physiologic relevance of GnRH-R changes to gonadatrophin secretion *in vivo* in mice is uncertain, but the receptor is probably not a major regulatory locus for hormonal control of gonadotrophin release. Modulation of as yet undefined post-receptor events are probably or more importance. Only during the replacement of GnRH to *hpg* mice is there a clear suggestion of a casual link between GnRH-R and gonadotrophin synthesis and secretion. Even in this situation, in which receptors are much reduced, a single injection of GnRH is capable of releasing a small amount of LH, attesting to the functional coupling of the limited number of receptors. It is probably that in *hpg* mice the GnRH receptor increase is yet another manifestation of the trophic action of GnRH on the gonadotroph, rather than a prerequisite for the induced gonadotropin response.

At first sight it appears that the post-castration GnRH-R changes in mice are at variance with the homologous ligand up-regulation hypothesis for hormonal regulation of GnRH-R *in vivo*. However, the only modification needed is to include a requirement for gonadal steroids as an essential component for receptor maintenance, and expression of up-regulation by GnRH.

GnRH RECEPTOR REGULATION *IN VIVO* IN OTHER SPECIES

A number of studies have been reported of GnRH-R changes in other rodent (hamster) and non-rodent species (Table 5). In cows, sheep, and hamsters gonadectomy increases GnRH-R [26, 27]. Lactation in the hamster is also associated with a receptor and serum LH fall, as in rats and mice, and the pattern of GnRH-R changes during the hamster estrous cycle parallels that of the rat [28]. No studies to date have described GnRH-R changes during the mouse estrous cycle.

In ovariectomised rhesus monkeys estradiol treatment causes an initial GnRH receptor and serum LH suppression (6–12 h), followed by a GnRH-R increase of >100% about 30 h post-E_2, coincident with the E_2-induced LH surge [29]. Thus, in both non-primate and primate species estrogen induces GnRH-R prior to and at the time of the preovulatory LH surge. It is tempting to ascribe the E_2-induced enhanced pituitary sensitivity to GnRH at least in part to the higher number of GnRH-R, though this remains to be proven. On the other hand the GnRH-R rise may merely reflect the general stimulatory effect of estrogen on protein synthesis.

In the male golden (Syrian) hamster GnRH-R fall, in parallel with serum LH and FSH, during testicular regression on transfer from long (14L:10D) to short (5L:19D) days. This is associated with an increase in hypothalamic GnRH content [30]. During spontaneous testicular recrudescence in continuing short days, or on transfer from short to long days increases in GnRH-R accompany the rise in serum FSH. These changes most likely reflect the alterations in endogenous GnRH secretion which are known to vary with photoperiod in this and other species. Therefore, the golden hamster seems to depend largely upon a direct action of GnRH for its pituitary receptor regulation, as does the rat. Similarly, GnRH-R regulation in other non-rodent species resembles that of the rat rather than the mouse (cf. gonadectomy—Table 5). Thus, the concept that GnRH itself is the major hormonal regulator of GnRH receptors seems widely applicable across many species, and even in the mouse a role for GnRH in receptor maintenance has been demonstrated.

GnRH RECEPTOR REGULATION *IN VITRO*

Introduction

Enzymatically dispersed pituitary cell cultures have been employed for over 12 years as bioassays for hypothalamic releasing and inhibiting factors. More recently their use has been extended to the investigation of the cellular biochemical mechanism of GnRH action, including GnRH receptor regulation. Several advantages exist over *in vivo* animal models the major of which is the ability to study the effects of a precisely defined hormonal milieu, particularly the controlled exposure to GnRH itself, which fluctuates and is never precisely known in the intact animal. Additionally, cell cultures allow a precise definition of the relationship between GnRH receptor changes and subsequent responsiveness to the hormone.

The first report of GnRH analogue binding to pituitary cells [31] showed that the ligand bound to a single class of high affinity sites, with a very similar K_d (2×10^{-10} M) as to pituitary membrane preparations or homogenate. Furthermore, GnRH receptors are confined to the gonadotroph cells of the pituitary [32]. By comparison of the K_d for GnRH binding with the ED_{50} for LH release it is clear that occupancy of between 15–20% of receptors is sufficient to produce the maximum LH response [31]. Thus, there are a large number of "spare" receptors for GnRH, as also occurs for other hormones [5]. Following these initial studies a number of groups [33–36] have analysed the hormonal regulation of GnRH-R and related this to LH release.

HOMOLOGOUS LIGAND ACTION ON GnRH-R

In our own studies [36] we employed collagenase-dispersed adult female pituitary cells cultured in DMEM + 10% horse serum and 2.5% foetal calf serum. After 60 h in culture GnRH, or other stimuli, were added directly to the culture without medium change, or when examining the effect of Ca^{2+} the medium was changed and replaced with fresh serum-containing medium supplemented with 3 mM EGTA. After 8–10 h with the stimuli medium was removed and the intact cells were washed with PBS, scraped from the dish with a rubber policeman, and resuspended in a small volume of receptor assay buffer (PBS containing 0.1% BSA). From each treatment three maximum binding and two non-specific binding tubes were obtained containing approx 2×10^5 viable cells each. Receptor content was assessed by incubation with about 10^{-10} M [^{125}I]GnRH-A for 80 min at 22°C [31, 36]. We did not attempt to dissociate any residual GnRH bound to receptors after the treatment period [34], prior to their measurement. We found this unnecessary since we never observed any reduction in [^{125}I]GnRH-A binding even at early

Table 5. Pituitary GnRH receptors in physiological circumstances

	Rat	Mouse	Hamster	Sheep	Cow	Monkey
Oestrous cycle	↑	?	↑	?	↑	?
Oestrogen-induced	↑	?	?	<——>	?	↑
Lactation	↓	↓	?	?	?	?
Gonadectomy	↑	↓	↑	↑	↑	?

↑ = increased ↓ = decreased ? = not known <——> = unchanged.

times (1/2–4 h) after incubation of the cells with 10^{-9} M GnRH [36]. This is in contrast to the reports by Loumaye *et al.*[33, 37] and Conn *et al.*, [35] who showed an initial 30–40% decrease in available GnRH-R prior to the later receptor increase. We observed an increase in GnRH-R of between 70–150% from 6 and 12 h after a single exposure to 10^{-9} M GnRH, which returned to pre-treatment values by 16 h. In all subsequent studies we used a 7–10 h exposure time. Receptor up-regulation was observed with 10^{-10}–10^{-9} M GnRH but 10^{-8} M was less effective, probably because of continued receptor occupancy by the ligand. We also found that if cells were incubated with 10^{-9} GnRH for 10 h in medium *without* serum no receptor induction occurred. When we measured residual GnRH in the medium, with a specific radioimmunoassay, some 80% of that initially added remained, whereas no immunoreactive GnRH was detectable after incubation of cells for 10 h in serum-containing medium [21]. We feel that residual receptor occupancy or processing is the reason for the failure to observe up-regulation, which may possibly have been revealed if dissociation of ligand–receptor complexes had been attempted. Under no circumstances in this, and subsequent sections, could the increase of GnRH receptors be attributed to enhanced receptor affinity, as also applied *in vivo*.

GnRH RECEPTOR INDUCTION: PROTEIN SYNTHESIS AND MICROTUBULE DEPENDENCE

Receptor up-regulation by GnRH depends upon its interaction with its own receptors since this is blocked by concurrent incubation with a GnRH antagonist, which also blocks stimulated LH release [33, 35, 21]. Co-culture of 10^{-9} M GnRH with 9 μM cycloheximide, or the microtubule disrupting agent vinblastine (1 μM), effectively blocks the receptor increase, but neither agent inhibits the GnRH-stimulated release of LH during a 3 h incubation. This data indicates a clear dissociation between the biochemical pathways involved in receptor up-regulation and LH secretion, as was observed in mice *in vivo*. The dissociation also shows that new GnRH receptors are not inserted into the plasma membrane from the LH secretory granule membranes. These data do not enable any firm conclusions to be drawn regarding *de novo* receptor synthesis by GnRH, or whether cycloheximide blocks the synthesis of other proteins required for the process of receptor turnover. Probably a combination of events is involved since we observed a significant decrease in receptors to below untreated values when GnRH and cycloheximide were present together, both *in vitro* [21] and *in vivo* [22, 24].

MEMBRANE DEPOLARISATION AND GnRH RECEPTORS

Generalised membrane depolarisation with high concentrations of potassium (KCl, 58 mM) is a potent secretagogue in many secretory cell types, including the gonadotroph where LH release is of similar magnitude to that observed with maximal concentrations of GnRH. Like GnRH, 58 mM KCl increases GnRH-R by 100% or more after 8–10 h of exposure. As anticipated KCl-induced GnRH-R up-regulation was not prevented by the GnRH antagonist, although cycloheximide and vinblastine were effective in this regard. Thus, the final cellular events in GnRH and KCl-induced GnRH-R up-regulation are similar, if not identical.

CYCLIC ADENOSINE NUCLEOTIDES AND GnRH RECEPTOR REGULATION

The role of cyclic adenosine nucleotides (cAMP) in GnRH action is controversial. It has been claimed [38, 39, 40, 41] that cAMP is the "second messenger" for GnRH-stimulated LH release. However, others [42] have considered this unlikely since: (1) cAMP rises after GnRH stimulation are either small or if evident occur after the time of maximal LH release; and (2) there has been no consistent stimulation of adenylate cyclase by GnRH in pituitary membrane preparations. However, cAMP may well be involved in later actions of GnRH since exogenous active analogues (8 BrcAMP/DbcAMP) can increase LH glycosylation [43]. Moreover, exogenous cAMP derivatives increase FSH and LH receptors in rat granulosa cells as well as induce morphological differentiation of granulosa/luteal cells [44, 45]. Therefore, we considered that a possible action of cAMP in the pituitary would be GnRH-R induction.

Incubation of pituitary cells with 1 mM dibutyryl cAMP (DbcAMP) increased GnRH-R by 100% with an identical time-course as GnRH [36]. The DbcAMP effect was dose-dependent with as little as 0.3 mM producing a significant increase. 8-BrcAMP and cAMP itself (1 mM) were as effective as DbcAMP, while adenosine, cGMP and Na butyrate were ineffective. Interestingly, while treatment of the cells with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) alone did not alter GnRH-R, the combination of 1 mM DbcAMP + IBMX (0.2 mM) prevented receptor induction by the former agent. The inhibitory property of IBMX has also been observed with exogenous cyclic nucleotide induction of FSH receptors in granulosa cells [44], though the explanation of this phenomenon is unknown. Like GnRH and KCl, DbcAMP induction of GnRH-R was prevented by cycloheximide and vinblastine, and also by the GnRH antagonist. Smith *et al.*[41] have previously reported that DbcAMP-induced release of LH during a 4 h incubation could be blocked by a GnRH antagonist, which also inhibited [^{125}I]GnRH-A binding to rat pituitary homogenate at high concentrations (1–10 mM). They did not find any GnRH-R upregulation by DbcAMP, probably because the maximum incubation period was too short (4 h). We also observe that DbcAMP

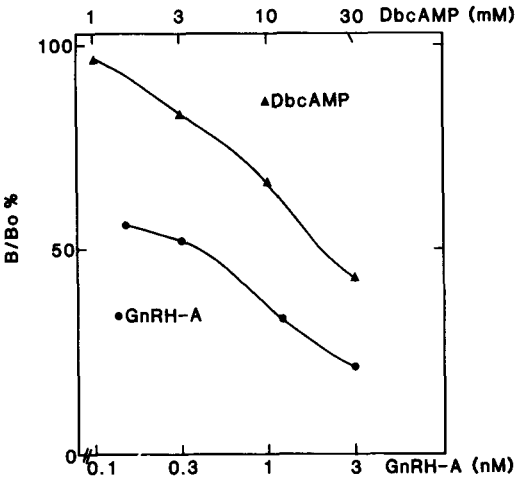

Fig. 2. Interaction of dibutyryl cAMP (DbcAMP) and a GnRH agonist analogue (GnRH-A) with GnRH receptors in cultured intact rat pituitary cells *in vitro*. Receptor assay performed as described in Young *et al.* Values represent specific [^{125}I]GnRH-A-binding and are mean of triplicate determinations expressed as a percentage of tracer binding ($\simeq 3 \times 10^{-10}$ M [^{125}I]GnRH-A) in the absence of competitor (B/B_0 %).

inhibits [^{125}I]GnRH-A binding to intact pituitary cells over the range of doses which induce GnRH-R (Fig. 2), and also in solubilised pituitary and ovarian GnRH receptor preparations [46]. However, it remains to be shown whether cyclic nucleotides bind to the same domain of the receptor as GnRH itself or to an adjacent domain of the plasma membrane thereby rendering the GnRH binding sites inaccessible to the ligand.

We next considered whether *endogenous* cyclic nucleotides could up-regulate GnRH-R. Attempts to increase GnRH-R with choleratoxin (10–100 ng/ml) were unsuccessful, although the treatment duration may have been too short (8–10 h) since we observed forskolin induced GnRH-R up-regulation at later times (v.i.). Direct stimulation of the adenylate cyclase catalytic unit with forskolin 1 μM did increase GnRH-R by 70% (Fig. 3A), although this was observed only after 24 h incubation and not at 6 h. Ten μM forskolin was without effect both at 6 or 24 h though cell viability (trypan blue exclusion) was not impaired. Thus, the time-course of GnRH-R up-regulation by endogenous cAMP seemed different from that with exogenous analogues.

When the LH releasing ability of exogenous DbcAMP was examined in a 3 h incubation we consistently observed a 2–3-fold stimulation over basal though this was never as much (6–10-fold) as with either 1 mM GnRH or 58 mM KCl. We also found that the GnRH antagonist could block the slight stimulation of LH release seen with DbcAMP [41]. In contrast to the absence of an effect of 10 μM forskolin on GnRH-R, 3 h incubation with this concentration doubled LH release though the effect at 24 h was more pronounced (5-fold stimulation, Fig. 3B). One μM forskolin slightly increased LH release after 6 h but at 24 h was nearly as effective as 10 μM forskolin. These effects of forskolin on LH release are in agreement with those recently reported [47]. Again, the forskolin data demonstrates the dissociation between the agent's action on GnRH-R and hormone secretion *in vitro*.

THE ROLE OF CALCIUM IN GnRH-R REGULATION *IN VITRO*

As reviewed by Conn *et al.*[48], increased intracellular calcium (Ca^{2+}) is obligatory for GnRH-stimulated LH release. Conn *et al.*[35] have also shown that GnRH-R up-regulation, but not down-regulation, *in vitro* is dependent upon Ca^{2+}. In our own studies we investigated the Ca^{2+} dependence of GnRH receptor up-regulation by GnRH (1 nM), KCl (58 mM), and DbcAMP (1 mM). The presence of EGTA (3 mM) in the medium (which was not Ca^{2+}-free) prevented GnRH-R induction and LH release by all three agents, as did the Ca^{2+} channel blocker verapamil (0.1 mM). These data confirm

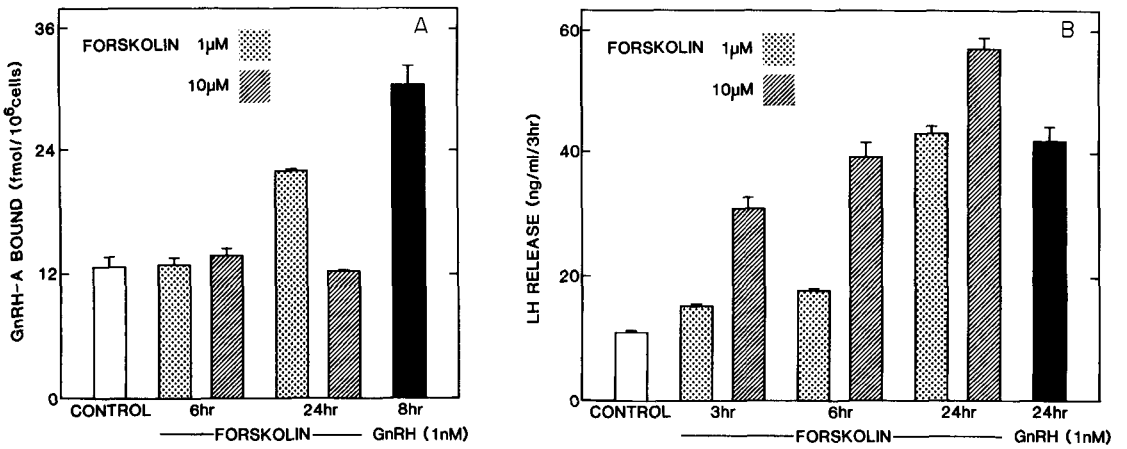

Fig. 3. Effect of incubating cultured pituitary cells with forskolin (Sigma) and GnRH for various times on GnRH receptors (A) and LH release (B). Cells were prepared, cultured, and treated as described in Young *et al.*, 1984. Values represent the mean ± SE for triplicate cultures with each treatment from the same dispersion.

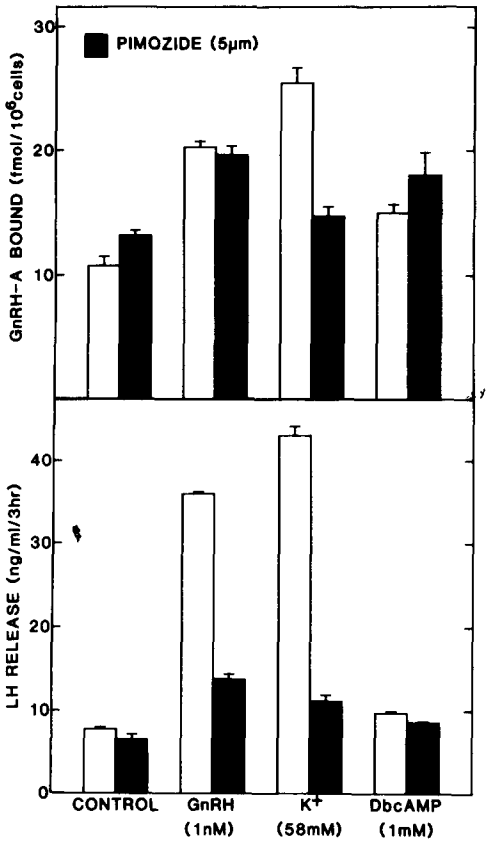

Fig. 4. Effect of treating cultured pituitary cells for 10 h with GnRH, KCl (K^+), or dibutyryl cAMP (DbcAMP) in the presence (■) or absence of pimozide (□) on GnRH-R (upper panel), and LH release over 3 h (lower panel). Values are the mean ± SE of triplicate cultures from the same dispersion and methods were as described in Young *et al.*, 1984.

those of Conn *et al.*[35] and Loumaye and Catt[37]. Furthermore, the Ca^{2+} ionophore, A23187, at low concentrations (0.01 and 0.1 μM) doubled GnRH-R *without* stimulating LH release, while higher concentrations had either no effect (1 μM) on GnRH-R or reduced these by 50% (10 μM), though LH release was increased 4–5-fold over basal. 8–10 h incubations with EGTA (3 mM) verapamil (0.1 mM) and A23187 (0.1 and 10 μM) did not damage the cells as judged by normal dose–response curves to subsequent GnRH stimulation.

Since Ca^{2+}-induced LH release appears to be dependent upon activation of calmodulin dependent enzymes [49] we examined the effect of the calmodulin inhibitor, pimozide. As shown in Fig. 4 pimozide (5 μM) had no effect on either GnRH or DbcAMP stimulated GnRH-R increases, though the increase by KCl was almost completely prevented. Nevertheless, pimozide markedly inhibited LH release in response to both KCl and GnRH. These results have been reproducible over several experiments. Thus, whilst it was clear that Ca^{2+} is essential for GnRH-R up-regulation by all agents examined it seems that only KCl depends upon calmodulin for this effect. Perhaps this indicates that the changes in intracellular Ca^{2+} evoked by GnRH and cAMP analogues activate different enzyme systems. Phospholipid-Ca-dependent protein kinase C, which is now thought to be a key enzyme in signal transduction by many surface receptor ligands which are not coupled to adenylate cyclase, has been implicated in stimulation of LH release by GnRH [2]. However, our preliminary results with the cocarcinogen TPA (tetradecanoylphorbol acetate) failed to show any GnRH receptor increase, although there was no release of LH either, indicating either an inappropriate dose or time of exposure, or an inactive preparation.

RELATIONSHIP BETWEEN GnRH RECEPTOR CHANGES AND GONADOTROPHIN RESPONSES

As has been stressed in the previous sections a divergence between GnRH-R and LH responses to hormonal manipulations *in vivo* and *in vitro* is readily apparent.

In Table 6 are summarised respective changes in GnRH-R and LH release in response to various agents *in vitro*.

We have also determined whether an increase in GnRH-R has any effect on the sensitivity of gonadotrophs to a subsequent challenge with varying doses of GnRH. Cells were treated for 10 h with either GnRH (1 nM), KCl (58 mM), or DbcAMP (1 mM), washed extensively, and then incubated for 3 h with fresh GnRH (10^{-10}–10^{-8} M) and LH release determined. The GnRH-R content of the treated cells was double that of the untreated controls, but despite this there was no reduction in the ED_{50} of GnRH, i.e. no shift to the left in dose–response curves, after any pretreatment. The maximum LH released by 3×10^{-9} M GnRH was reduced by pretreatment with either GnRH or KCl, but not with DbcAMP. This decrease in maximum output could be entirely accounted for by depletion of cellular LH content during the pretreatment period. It could be argued that the pretreatment period caused a specific defect in the "GnRH-releasable" pool of LH. However, if cells receiving identical pretreatments were subsequently challenged with increasing concentrations of Ca ionophore (A23187, 1–100 μM) or KCl (5–100 mM) there was no change in sensitivity, al-

Table 6. GnRH-R up-regulation versus LH release *in vitro*

Treatment	GnRH-R*	LH release†
GnRH (1 nM)	↑↑↑	+ + +
KCl (58 mM)	↑↑↑	+ + +
DbcAMP (1 mM)	↑↑↑	±
‡Forskolin (1 μM)	↑↑↑	+ +
(10 μM)	←——→	+ + +
A23187 (0.1 μM)	↑↑	—
(10 μM)	↓↓	+ + +
Oestradiol (1 nM)	↑	–

*Measured after 7–10 h exposure unless otherwise indicated.
†Measured during 3 h exposure unless otherwise indicated. Number of +/↑ indicates magnitude of response.
‡24 h exposure for both GnRH-R and LH release.

though the maximum LH output was decreased according to the extent of cellular LH depletion. These data mean that either (1) any change in sensitivity is so small as to be undetectable by these methods; or (2) the increased GnRH receptors are not functionally coupled to the LH release machinery of the cell after 10 h. We favour the latter explanation, and perhaps repetitive treatments with smaller doses of GnRH over a period of days may be required for effective coupling of the "new" receptors.

The only situations wherein changes in GnRH-R are accompanied by alteration in subsequent sensitivity to GnRH occur following gonadal steroid treatment. Thus, Giguere *et al.*[50] were able to show that dihydrotestosterone treatment for 48 h reduced GnRH-R by 40% and shifted the GnRH dose–response curve to the right. Estrogen, on the other hand, consistently sensitises pituitary cells to GnRH with a reduction in the ED_{50} from between 2–10-fold [51, 52] although GnRH-R after E_2 treatment [53, 54] show only a modest increase (25–50%). We have been unable to show a consistent effect of estradiol-17β (1–100 μM) on GnRH-R in cultured cells, even though the E_2-exposure time has varied from 12–72 h (unpublished). These same cells show a small shift (2–3-fold) to the left in the GnRH dose–response curve.

Therefore, these *in vitro* data, as well as the *in vivo* data in mice, imply that regulation of the GnRH receptor is not a major locus for hormonal modulation of gonadotroph sensitivity. This is largely conferred by changes in post-receptor cellular events which remain to be clarified. This same conclusion was reached by Clayton[19] and Smith *et al.*[34] in respect of the mechanisms of pituitary desensitisation *in vivo* and *in vitro*, respectively. In their studies, net loss of GnRH receptors was a minor component of the desensitisation phenomenon. While these authors could show a considerable degree of receptor occupancy by GnRH, receptor coupling to the secretion process was clearly disrupted.

CONCLUSIONS

With the ready availability of synthetic GnRH a wealth of information has become available on its biological actions. At a cellular level within the pituitary the mechanism of GnRH action has been extensively investigated. GnRH receptors do not appear to be coupled to the adenylate cyclase enzyme, and much evidence indicates a requirement for Ca^{2+} in GnRH activated LH release. The very early cellular events in GnRH action may involve membrane phospholipid metabolism and possible involvement of diacylglycerol stimulation of protein kinase C, an area currently under investigation. Ability to study the physiological and hormonal regulation of the GnRH receptor itself has led to the conclusion that this receptor is under homologous ligand up-regulation and down-regulation. The biochemical mechanisms are different for the two processes, the former being calcium dependent, the latter not so. *In vivo*, there is considerable species similarity in the GnRH receptor response to altered gonadal hormone milieu, though this is not invariably the same, as evidenced in mice. In female mice maintenance of a "normal" GnRH-R complement depends upon estrogens acting together with GnRH. In normal female mice there is a marked dissociation between GnRH receptors and basal serum gonadotrophins indicating a major role for post-receptor sites in hormonal regulation of gonadotrophin secretion. The same conclusion is derived from *in vitro* studies. Thus, while GnRH receptor changes can be demonstrated in many circumstances both *in vivo* and *in vitro* their relationship to gonadotroph function is clearly complex. It must be remembered that the RRA does not distinguish between functional and non-functional receptors and a method for measurement of functionally coupled receptors might reveal a closer correlation between receptor regulation and cellular activation.

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