GnRH and oxytocin have nonidentical effects on the cellular LH response by gonadotrophs at pro-oestrus

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Abstract

For full fertility in the female ovulation is necessary, which is dependent on the production of a surge of LH. An understanding of the processes which result in the high levels of LH requires delineation of the activities of individual component cells. In this study the responses of gonadotrophs to two signalling hypothalamic peptides, GnRH and oxytocin, were investigated. A cell immunoblot method was used to identify and distinguish between cells which secrete LH and those which contain LH but do not secrete the glycohormone. Rats were killed on the morning of pro-oestrus, the pituitary collected and the cells dispersed onto a protein-binding membrane for study. Cells were then incubated with GnRH and oxytocin, after which the membranes including the attached cells were stained by immunocytochemistry for LH. GnRH increased the total number of immunopositive cells which were present in a concentration-dependent manner. The most prominent change after 2 h incubation was in the number of secreting cells, whereas after 4 h there was also a marked increase in numbers of nonsecreting cells. Oxytocin also increased the total number of immunopositive cells in a concentration–responsive manner, however the profile of action of oxytocin was different from that observed for GnRH. Oxytocin had a relatively greater effect on numbers of immunopositive nonsecreting cells. Thus, the results reveal the potential for gonadotrophs to be flexibly and appropriately modulated by selected hypothalamic peptides. When cells were pre-incubated with oxytocin prior to GnRH there was not an additive increase in the numbers of immunopositive cells, suggesting that the two agonists act, in a nonidentical manner, on similar cells. The increase in the total number of immunopositive cells implies that there was a production of LH or post-translational processing, induced by exposure to GnRH or oxytocin. The results confirmed the heterogeneity of gonadotrophs and the existence of functionally distinguishable subpopulations, and revealed a difference between the effects of GnRH and oxytocin on expression and secretion of LH.

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Introduction

The production of the regular ovulatory cycle is a result of the interaction of many co-ordinated factors. It is known, for example, that there are regular fluctuations in ovarian steroids and ovarian proteins. An important process is the preovulatory luteinising hormone (LH) surge which is preceded by a surge of gonadotrophin-releasing hormone (GnRH) from the hypothalamus (Sherwood *et al.* 1980). However, the detailed cellular response of the functional pituitary to the exposure to GnRH, either endogenous or exogenous, is only scantily understood. For example, pituitary responses to GnRH are altered as a function of the oestrus cycle, and the ways in which the characteristics of gonadotrophs change is still to be established. One of the main problems in defining the response of the pituitary is the now well recognised heterogeneity of the gland. Not only is it made of several classical secretory cell types, but also within the population of gonadotrophs there are a number of subpopulations which can be defined on the basis of function.

Differences between gonadotrophs were noticed many years ago, including differences in size (Denef *et al.* 1978, Childs *et al.* 1992*a*), morphology (Childs *et al.* 1980), whether LH and/or FSH were present in a cell (Dada *et al.* 1983), physical density (Torronteras *et al.* 1993*b*), and ultrastructural characteristics (Torronteras *et al.* 1993*a*, Malagon *et al.* 1998). There was a change in density of LH staining within cells during the cycle (Childs *et al.* 1987), presumably reflecting secretion patterns culminating in the LH surge. It was suggested that gonadotrophs change among being monohormonal or bihormonal, and reserve, storage or secretory cells as parts of a cycle (reviewed in Childs 1995).

In vitro, gonadotrophs respond to GnRH by secreting LH and by increasing intracellular LH stores. A fuller

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understanding of the manner in which pro-oestrus gonadotrophs behave in the pituitary *in vivo* prior to the preovulatory surge has been limited by insufficient knowledge of the manner in which individual cells, from single rats, respond under particular circumstances. The regulated progression of the ovulatory cycle may be dependent on the ratios of cells which secrete LH in response to GnRH and those which do not.

In addition, the pituitary response is regulated by several factors besides GnRH. A number of other hypothalamic peptides, in particular oxytocin (Evans 1996), have also been observed to alter gonadotroph behaviour. Pharmacological blockade of oxytocin inhibits the preovulatory LH surge in female rats at pro-oestrus (Johnston & Negro-Vilar 1988, Robinson & Evans 1990). *In vitro* oxytocin increases secretion of LH from dispersed pituitary cells and stimulates an increase in intracellular Ca^{2+} in both dispersed normal pituitary cells and the αT31 cell line (Billiard 1996, Evans *et al.* 1997). However, oxytocin and GnRH do not have identical activities. Oxytocin is less potent than GnRH in stimulating secretion of LH. There are also differences in the manner in which GnRH and oxytocin induce desensitisation. Oxytocin reduces responsiveness to the homologous peptide more rapidly than GnRH (Evans *et al.* 1997). Oxytocin is also known to induce synthesis of LH in pituitary cells (Robinson *et al.* 1992). To our knowledge there are no studies on the response of individual pro-oestrus cells to oxytocin.

This study investigated the manner in which individual gonadotrophs respond to GnRH or oxytocin by comparing the numbers of cells which secreted LH and those which contained LH but did not respond by releasing stores. A cell immunoblot method, based on immunohistochemical techniques, was employed. The method is well established (Kendall & Hymer 1987, Arita 1993) and has been used for several applications at the pituitary, including the detection of prolactin (Kendall & Hymer 1987, Arita *et al.* 1991), substance P (Arita *et al.* 1993), ACTH (Perez *et al.* 1997) and FSH (Noguchi *et al.* 1996). In this study we have examined the regulation of LH. The method labels both LH in cells, and LH secreted from cells and adhering to a protein binding membrane. By visualising and counting nonsecreting and secreting cells we have been able to observe specific and nonidentical effects of two agonists (GnRH and oxytocin) on individual gonadotrophs.

Materials and Methods

Female rats were kept in controlled conditions with a ratio of 14 h light:10 h darkness (lights on 0500 h, off 1900 h). The studies were carried out in accordance with the principles and procedures of the animal ethics committee of the Christchurch School of Medicine. Oestrus cycles were monitored by cytology of vaginal smears taken daily. Rats which had had at least three consecutive 4-day oestrus cycles were used and were killed at 0900 h on the day of pro-oestrus. Pituitaries were collected and diced in medium consisting of 500 ml DMEM including 0·11 g sodium pyruvate, 4000 mg/l glucose (Gibco BRL Products, Gaithersburg, MD, USA) supplemented with 1·8 g Hepes, 1·5 g BSA, 800 U penicillin, 800 μg streptomycin and 1 mg amphotericin. The tissue was digested in the above medium (1 ml) with 3 mg trypsin and 1 mg DNase, shaking at 37 °C for 13 min. The partially dispersed cells were triturated in the medium (5 ml/pituitary) with 10 mg trypsin inhibitor, 1 mg DNase. The cells were filtered through gauze, washed and counted and viability assessed with trypan blue. Cells were suspended in Medium 199 (Gibco BRL Products) with Hanks' salts and L-glutamine, containing in 1 litre 1·40 g sodium bicarbonate, 5·95 g Hepes (Sigma–Aldrich, Castle Hill, Australia), 1 g BSA, 10 ml penicillin/streptomycin (Sigma–Aldrich) at pH 7·4.

For the cell immunoblotting procedure $1{\cdot}5 \times 10^4$ cells in a 80 μl droplet were added to a square of Immobilon P membrane (Millipore Corporation, Bedford, MA, USA). The cells were allowed to settle for 30 min, after which time test agent (GnRH or oxytocin) was added in 8 μl to the droplet to give final concentrations of 0·1–100 nM. When incubation was performed with oxytocin in combination with GnRH, the GnRH was added 30 min after oxytocin to the same droplet. The membranes were incubated in a humidified chamber at 37 °C for 2 or 4 h. Following the designated time the spent medium was discarded, the cells were washed and 100 μl 2·5% glutaraldehyde added as fixative. The fixed cells were washed (Tris buffer, pH 8·2) and incubated overnight in Tris buffer containing 3% BSA.

For staining, primary antibody (anti-LH raised in guinea-pigs; National Hormone and Pituitary Program) was added, followed by secondary antibody (anti-guinea-pig IgG coupled to biotin; Sigma–Aldrich). The site of LH immunoreactivity was visualised using avidin coupled to alkaline phosphatase, and substrate which was converted to a red product (Vector Laboratories, Burlingham, CA, USA). The membranes were covered with a cover slip over glycerol. The cells were viewed and counted with an oil immersion lens. Controls in which the primary antibody or the secondary antibody were omitted or which used primary antibody which was preadsorbed with LH indicated the specificity of the staining protocol.

Cells containing LH stained red (Fig. 1*a*). Secretion was observed as a region of red staining of membrane-bound protein outside a cell (Fig. 1*b*). Cells were counted manually by investigators blinded to the treatment of the cells. An average of 300 cells were counted per membrane, using an eyepiece grid and serpentine counting. Cells which exhibited secretion were counted into a category separate from those which were immunopositive but for which no staining outside the cell was seen. The sum of

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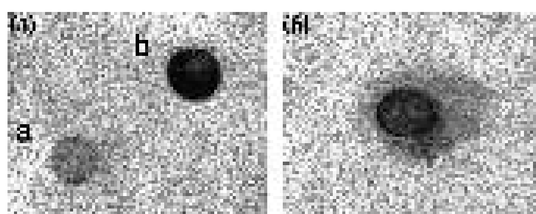

Figure 1 *a*: A cell which did not stain for LH (a) and an LH-immunopositive cell (b). *b*: An example of a cell which secreted LH. The stained secretion is seen as a dark area around this cell.

the two categories produced the total of immunopositive cells. The stained cells were calculated as a percent of the total number of cells on the membrane. Thus, percentage of immunopositive cells which were nonsecreting, and which were secreting, and total immunopositive cells were determined. A set of experiments utilised one pituitary, each condition being examined on usually four different blots and the means determined. Each experiment was performed at least three times on different rats, and the means ± S.E.M of the treatments were calculated. Comparison between groups was performed using one way ANOVA, and the appropriate Tukey test or Kruskal–Wallis analysis of variance on ranks. $P<0{\cdot}05$ was taken to indicate statistically significant differences.

Results

GnRH

After 2 h of exposure to GnRH, the total number of LH-immunopositive cells increased significantly. The response occurred in a concentration-dependent manner such that higher concentrations of GnRH induced the appearance of more immunopositive cells (Fig. 2). By separately noting the effect on numbers of secreting cells and nonsecreting cells more information could be obtained regarding the response of individual cells to GnRH. Although there was a small change in the numbers of nonsecreting cells, there was a larger increase in secreting cells induced by the GnRH. The effect was significant at concentrations of 1 nM and above, reinforcing the notion that the response is concentration-dependent.

The effects of a 4 h incubation were different to those of the 2 h incubation. At 4 h GnRH also markedly increased the numbers of nonsecreting cells. The differences in the percent of nonsecreting cells between the two times were statistically significant at 1 nM ($P=0{\cdot}02$) and 10 nM ($P<0{\cdot}05$) GnRH. This suggests a time-dependent effect of GnRH to increase the levels of LH within certain responsive cells. On the other hand, there were no significant differences between numbers of secretory cells after 2 h compared with 4 h at each concentration.

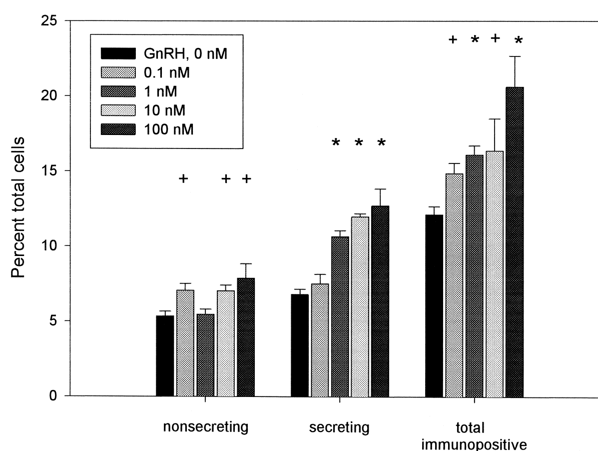

Figure 2 Cells were incubated without GnRH and with selected concentrations of GnRH for 2 h. The percent of total pituitary cells which contained LH but did not secrete LH, the percent of total cells which secreted LH and the total number of immunopositive cells were calculated. The means ± S.E.M. are depicted. Compared with incubations without GnRH: $^{+}P<0{\cdot}05$; $^{*}P<0{\cdot}002$.

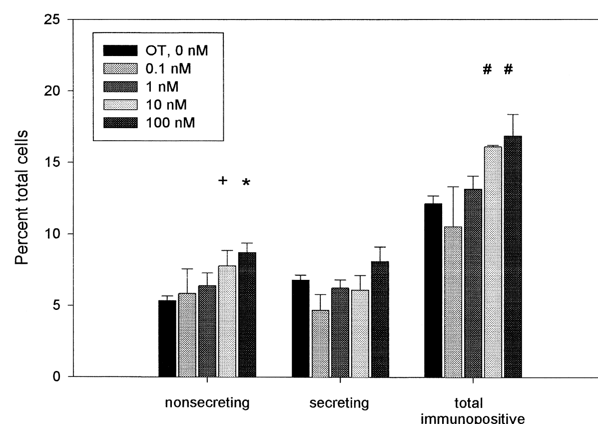

Figure 3 Cells were incubated without oxytocin (OT) and with selected concentrations of oxytocin for 2 h. The percent of total pituitary cells which contained LH but did not secrete LH, the percent of total cells which secreted LH and the total number of immunopositive cells were calculated. Compared with incubations without oxytocin: $^{+}P<0{\cdot}05$; $^{\#}P<0{\cdot}01$; $^{*}P<0{\cdot}002$.

Oxytocin

In a similar manner we investigated the effects of oxytocin. During a 2 h incubation oxytocin increased the numbers of immunopositive cells (Fig. 3). In contrast to the effect of GnRH at 2 h, oxytocin had a relatively lesser effect on secreting cells, and a prominent effect on increasing the number of immunopositive nonsecreting cells. At 4 h exposure oxytocin also increased the total numbers of immunopositive cells (data not shown).

GnRH plus oxytocin

The effect of adding GnRH to oxytocin was investigated with 2 h incubations (Fig. 4). Preincubation with oxytocin

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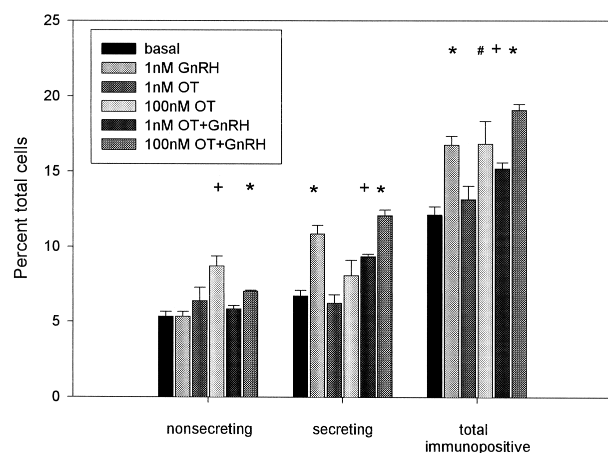

Figure 4 Cells were incubated with GnRH (1 nM), with oxytocin (OT, 1 or 100 nM) or a combination of oxytocin and GnRH, as described in Methods, for 2 h. The percent of total pituitary cells which contained LH but did not secrete LH, the percent which secreted LH and the percent of total cells which were immunopositive cells were calculated. Compared with incubations without peptide: $^{+}P<0{\cdot}05$; $^{\#}P<0{\cdot}01$; $^{*}P<0{\cdot}002$.

has been previously shown to affect the secretory response to GnRH (Evans *et al.* 1995). In this study when cells were preincubated with oxytocin prior to stimulation with GnRH there was no significant increase in the number of total immunopositive cells over the numbers seen when cells were incubated with GnRH alone. However, GnRH added to oxytocin produced a shift in the distribution of immunopositive cells, such that there was an increase in the number of secreting cells (oxytocin plus GnRH vs oxytocin, $P<0{\cdot}05$), and a decrease in the number of nonsecreting cells.

Discussion

The cell immunoblot method provides a sensitive and specific analysis of the response of individual cells to stimulation by gonadotrophin-regulating peptides. The total numbers of immunopositive cells in pro-oestrus pituitaries incubated in basal conditions was similar to that observed in previous studies using conventional methods of immunocytochemistry (Childs *et al.* 1987, 1992*b*, 1997). The cells prepared for the protocol of this study were functional as indicated by the responses to agonist peptides. The cell immunoblot method employs acutely prepared cells which are not subject to culture artefacts, such as those observed in cells cultured for more than 48 h which lose differential properties that were acquired *in vivo* (Tomic *et al.* 1994). Thus, this method has the ability to study behaviour of dispersed cells which reflect physiological characteristics, and these results and their relationship to others must be interpreted with the particular properties of the methods in mind.

The cell immunoblot has the advantage over most other methods in being able to assign secretion to individual cells, rather than population averages. The reverse haemolytic plaque assay (RHPA) is another method to detect secretion by individual cells. However, the RHPA may have a different detection profile than the present cell immunoblot technique. The RHPA method may be less sensitive than the current method because the numbers of immunopositive cells typically reported (Smith *et al.* 1984, Wei *et al.* 1995) are less than in the present investigation and in other studies using traditional immunocytochemical methods (Childs *et al.* 1987, 1992*b*, 1997). In addition, whereas RHPA uses aliquots on separate plates to consider LH within a cell and secreted LH (Lewis *et al.* 1989, Leong & Thorner 1991, Cassina *et al.* 1995), the cell immunoblot method can be used to determine whether an individual immunopositive cell also secretes LH. For the first time we have studied LH from this perspective.

Numerous previous studies on the average response of whole populations have documented that when GnRH is added to dispersed pituitary cells there is an increased release of LH. The present study revealed that after 2 h incubation there was an increased number of secreting cells, suggesting therefore that the increase in secretion is due, at least in part, to a recruitment of more cells to the subpopulation of secreting cells than were present in the control incubation. The results are consistent with some previous observations. An increase in secreting cells in response to GnRH has been observed also using RHPA on pituitaries from pro-oestrus rats (Lewis *et al.* 1989). GnRH was also observed to increase numbers of secreting cells in cells fractionated by elutriation from rats at mixed cycle stages (Lloyd & Childs 1988). The present study exploited the properties of the immunoblot method to obtain information on the cellular responses of cells from single rats at a defined stage of the ovulatory cycle.

The present results reveal also that there is a heterogeneity among the group of potential LH-secreting cells of pro-oestrus, because some have an apparently higher threshold of activation than others, as revealed by the concentration-sensitive nature of the responses. Our observations are consistent with some previous observations (Lewis *et al.* 1989, Leong & Thorner 1991) but contrast with some others, using RHPA, in which virtually all cells were secreting (Smith & Neill 1987). In fact, our studies provide further support for the hypothesis that response thresholds are a major means of regulating responses (Leong & Thorner 1991).

Given the observations of biological heterogeneity (Denef *et al.* 1978, Childs *et al.* 1980, 1992*a*, Dada *et al.* 1983) it can be inferred that some cells develop different intracellular responses to other cells. In one study only a proportion of gonadotrophs stained with anti-protein kinase-C (PKC) antiserum (Garcia-Navarro *et al.* 1991) and in others the Ca^{2+} response to GnRH was not equal in all gonadotrophs (Leong & Thorner 1991), and varied in

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cells during the cycle (Tomic *et al.* 1994). A recent study has revealed that increased intracellular cAMP levels enhance release of LH in some gonadotrophs, and inhibit in others (Cassina *et al.* 1995). These observations indicate the existence of subpopulations separable on the basis of intracellular processes which are operating.

In this study a static culture system was used. Although pulsatility is not achieved with this technique it allows accumulation of secreted product on the membrane. The concentrations of both GnRH (0·1–1 nM) (Sherwood *et al.* 1980, Ching 1982) and oxytocin (1–10 nM) (Sarkar & Gibbs 1984, Tannahill *et al.* 1988) have been measured in portal blood, but the actual levels at a target cell *in vivo* are uncertain since there is local production of both peptides (Morel *et al.* 1988, Pagesy *et al.* 1992) and possibly concentrating mechanisms.

Our study noted, with 2 h exposure to GnRH, only a small change in numbers of nonsecreting cells, but a large increase in secreting cells and an increase in the total number of immunopositive cells, relative to control incubations. The increase in immunopositive LH, which is necessary to produce the additional numbers of stained cells, is consistent with observations in which increases in immunoreactive LH have been observed after incubating pituitary cells with GnRH (Khar *et al.* 1978, Ramey *et al.* 1987, Stojilkovic *et al.* 1988). Whether this involves transcriptional processes is unclear (Liu & Jackson 1978, Haisenleder *et al.* 1991). Alternatively, the increase in immunopositive cells may reflect post-translational processing including translocation of secretory granules to the outer marginal region of the cytoplasm (Lewis *et al.* 1986). The observation of changes in cell characteristics indicates that GnRH can modulate the relative proportion of secreting and nonsecreting cells of pro-oestrus pituitaries. The method may have not detected some cells with localised secretion at a surface distant from the blotting membrane. Following 4 h incubation of pituitary cells with GnRH there was also an increase in the nonsecreting cell subpopulation.

Therefore, GnRH induces both the appearance of immunopositive material and secretion of immunopositive LH from gonadotrophs. This implies either that cells which were not previously immunocytochemically identified as gonadotrophs convert from another cell type, such as somatotrophs, or were gonadotrophs with low levels of LH which were not detected, or were FSH (single gonadotrophin)-containing cells (Childs 1995). It certainly appears that in pituitary cells demarcations between functional types are not as clear as the traditional model suggested. Indeed, the term somatogonadotroph has been used to describe cells which develop in such a manner (Childs 1995). However, such phenotypic interconversion is possibly more likely to occur within the context of a 4-day oestrus cycle than during a short term *in vitro* incubation. The possibility, that GnRH stimulates the conversion of monohormonal cells to bihormonal, is strongly suggested by previous observations (Childs 1985, Childs *et al.* 1994). Thus, considerations of the production of the LH surge will, in a complete version, incorporate the relationship of LH with follicle-stimulating hormone (FSH) and other factors. The relationship between immunopositive nonsecreting and secreting cells is a fascinating issue.

Additionally, and importantly, it was observed that oxytocin increased the numbers of immunopositive cells. The role of oxytocin in regulation of the ovulatory cycle is still uncertain although it is well established that oxytocin increases release of LH from dispersed anterior pituitary cells and can interact with GnRH *in vivo* (Evans & Tulloch 1995) and on tissue pieces *in vitro* (Evans *et al.* 1995). The present increase in immunopositive cells induced by oxytocin reflected an increase more in nonsecreting than secreting cells. It should be borne in mind that oxytocin had a relatively greater positive effect than did GnRH on the subpopulation of immunopositive but nonsecreting cells. This observation is consistent with previous reports that oxytocin is a less potent LH-secretagogue than GnRH. Nevertheless, oxytocin is known to have effects on gonadotrophs, as has been observed with increased inositol phosphate production in αT3–1 cells (Evans *et al.* 1997) and increased Ca^{2+} levels in both αT3–1 cells and in mature gonadotrophs (Billiard 1996, Evans *et al.* 1997). Thus, the responsiveness of gonadotrophs to oxytocin is well established. However, the details of the effects of oxytocin on gonadotrophs as they might relate to the physiological condition are uncertain. The results here are consistent with a previous suggestion that oxytocin might be involved at an earlier stage of the production of the LH surge than GnRH, preparing the pituitary for the arrival of the potent LH secretagogue (Evans *et al.* 1995). The results suggest that one of oxytocin's physiological roles in the ovulatory cycle may be to increase stores of releasable LH in cells. The LH would be available to subsequent secretory stimulation by GnRH.

In this study GnRH and oxytocin together did not increase the numbers of immunopositive cells in an additive manner. These results imply that the two agonists act at least partly on the same cells, a result consistent with observations obtained by examining intracellular process in the αT3–1 cell line (Evans *et al.* 1997). However, the profile of cell response to the mix of peptides was different to that produced by either GnRH or oxytocin alone. This investigation revealed that there are potential complementary processes induced by agonists at the pituitary enabling sequential or concurrent pathways involving hypothalamic peptides to be incorporated into the regulatory mechanisms. These activities must be co-ordinated within the various processes that affect gonadotrophs, including priming, synthesis and desensitisation.

In summary, it was confirmed that GnRH will stimulate pituitary cells to secrete LH, with some individual cells being sensitive to lower levels of GnRH than others. This

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suggests that *in vivo* there are some gonadotrophs which will respond to the lower levels of GnRH at the early stages of the preovulatory surge and others which will not release LH until the GnRH surge is more fully developed. The results confirm therefore, that gonadotrophs are not a homogeneous population of cells. In addition, gonadotrophs responded to exposure to oxytocin. Because oxytocin also did not affect all gonadotrophs identically, there is potential for a complex, and therefore flexible, regulation of the pituitary during the ovulatory cycle.

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