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Estrogen Induces Neuropeptide Y (NPY) Y1 Receptor Gene Expression and Responsiveness to NPY in Gonadotrope-Enriched Pituitary Cell Cultures

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We showed previously that neuropeptide Y1 receptor (Y1R) expression is increased in the hypothalamus on proestrus afternoon and that this up-regulation of Y1R mRNA may permit neuropeptide Y (NPY) to facilitate release of the preovulatory GnRH surge. Because NPY also modulates LH release directly, we examined steroid regulation of Y1R expression in the female rat anterior pituitary. Treatment of female rats with estrogen *in vivo* decreased the levels of Y1R mRNA in the whole pituitary gland. In lactotrope/somatotrope-enriched pituitary cells separated by unit gravity sedimentation, 17β-estradiol (E_2) treatment likewise suppressed Y1R expression. In contrast, E_2 elevated Y1R mRNA in gonadotrope-enriched cell populations, indicating that estrogen regulates Y1R mRNA expression differently in gonadotropes *vs.* other pituitary cell types. After exposure to E_2, NPY augmented GnRH-induced LH release from gonadotrope-enriched cells in a manner requiring Y1R activation. Without steroid exposure, this augmentation disappeared, and with progesterone alone, NPY reduced GnRH-induced LH release. In addition, NPY inhibited prolactin secretion from primary pituitary cells in a steroid-free environment, but not in the presence of estrogen. These findings demonstrate that E_2 can directly up-regulate gonadotrope responsiveness to NPY and suggest that this action is mediated at least in part by E_2's ability to stimulate Y1R gene expression in gonadotropes. Our observations are consistent with the idea that this regulatory mechanism represents a component of E_2's positive feedback actions in pituitary gonadotropes. The biological importance of E_2's opposite effects on Y1R expression in other pituitary cell types remains to be determined. (*Endocrinology* 145: 2283–2290, 2004)

THE CENTRAL EVENT of the female reproductive cycle, ovulation, depends on the coordinated release of reproductive hormones and modulatory factors such as neuropeptide Y (NPY). Throughout the cycle, NPY accompanies the pulses of GnRH that are intermittently released into the hypophysial portal vasculature (1). During the afternoon of proestrus, an acute increase in endogenous NPY neurosecretion occurs in parallel with the rise in GnRH (2–4) during the initiation of the gonadotropin and prolactin (PRL) surges. This surge of NPY amplifies the ability of GnRH to trigger the preovulatory LH surge. The role of NPY in the regulation of PRL secretion is less clear.

NPY plays an obligatory role in the LH surge-generating process. Immunoneutralization of NPY in the portal circulation greatly attenuates the LH surge (4), and the LH surge is stunted in NPY-knockout mice (5). Numerous studies have shown that NPY augments LH release on proestrus (6–9), and pentobarbital-blocked, proestrous rats require both GnRH and NPY replacement for an LH surge of normal proportions (10). Importantly, this augmentation of LH release cannot occur without a proestrous hormonal environment. NPY augments GnRH-stimulated LH secretion from anterior pituitaries removed from proestrous, but not metestrous, rats (11). NPY also has no effect on *in vivo* GnRH-stimulated LH secretion in pentobarbital-treated, metestrous, or ovariectomized (OVX) rats (10). Notably, the actions of GnRH on LH release are attenuated in estrogen-primed NPY-knockout mice (5).

Studies demonstrate that NPY acts through the neuropeptide Y1 receptor (Y1R) subtype to augment LH release. Peripheral administration of the selective Y1R antagonist BIBP3226, a compound that does not cross the blood-brain barrier (12), attenuates both LH secretion in proestrous rats and surges induced by GnRH and NPY in pentobarbital-blocked, proestrous rats (9). Y1R expression is also highly dependent on circulating levels of steroid hormones. Y1R mRNA levels in the hypothalamus increase during the late morning and afternoon of proestrus, when 17β-estradiol (E_2) levels are high, and E_2 administration replicates this gain (13). In addition, Musso *et al.* (14) found that E_2 treatment induces Y1R gene expression in transfected neuroblastoma-glioma cells through the direct interaction of estrogen receptor α (ERα) with three hemipalindromic estrogen-responsive elements flanking the Y1R gene.

NPY may also influence PRL levels through actions at both the hypothalamic and pituitary level. Hsueh *et al.* (15) have shown that intracerebroventricular injection of NPY increases tuberoinfundibular dopaminergic neuronal activity levels and suppresses PRL secretion. Furthermore, this effect can be mimicked with a Y1R agonist and blocked by a Y1R

Abbreviations: DNase, Deoxyribonuclease; E_2, 17β-estradiol; EB, E_2 benzoate; ERα, estrogen receptor α; ERβ, estrogen receptor β; FBS, fetal bovine serum; NPY, neuropeptide Y; OVX, ovariectomized; P_4, progesterone; PRL, prolactin; S-MEM, suspension MEM; Y1R, neuropeptide Y1 receptor.

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antagonist. Injection of NPY also fails to increase tuberoinfundibular dopaminergic neuronal activity when Y1R synthesis is blocked in the medial preoptic area (16). Likewise, NPY administration to dispersed anterior pituitary cells has been reported to suppress PRL release and enhance the ability of dopamine and TSH-releasing hormone to suppress PRL release through a Ca^{2+}-dependent mechanism (17). Such data raise the question of how a robust PRL surge can occur on the afternoon of proestrus, when high concentrations of NPY exist in the hypophysial portal vasculature. In contrast, others have reported NPY augmentation of PRL gene expression at the male rat pituitary (18) and no effect of NPY on PRL secretion at the bovine pituitary (19).

In these studies, we tested the hypothesis that E_2 stimulation of Y1R expression in the gonadotrope allows NPY to facilitate LH release from these cells. Furthermore, we examined whether E_2 acts similarly on Y1R expression in other pituitary cell types, including those that may exhibit a contrasting response to NPY receptor activation, such as lactotropes (17). These experiments were carried out in dispersed cells to permit the examination of both receptor gene expression and hormone secretion in the same cell population.

Materials and Methods

Reagents

DMEM (Cellgro) was purchased from Mediatech, Inc. (Herndon, VA), fetal bovine serum (FBS) was obtained from Life Technologies, Inc. (Rockville, MD), and Tri Reagent and Tri Reagent-LS were purchased from the Molecular Research Center (Cincinnati, OH). Sesame oil, E_2 benzoate (EB), E_2, progesterone (P_4), bovine albumin (fraction V), pancreatin, collagenase II, deoxyribonuclease (DNase) II, MEM with D-valine, suspension MEM (S-MEM) powder, human NPY (identical to rat NPY), [Ala31,α-aminoisobutyryl(α-methyl-Ala)32]-NPY, rat pancreatic polypeptide, and Matrigel were purchased from Sigma (St. Louis, MO). *Taq* DNA polymerase was purchased from Fisher Scientific (Chicago, IL), and T7 RNA polymerase, RQ1 DNase I, deoxynucleotide triphosphates, RNase inhibitor, and Maloney murine leukemia virus-reverse transcriptase were supplied by Promega (Madison, WI). Dr. Stephan Mueller of Boehringer Ingelheim Pharma KG (Ingelheim, Germany) generously provided the BIBO3304TF.

Animal protocols

Female Sprague Dawley rats were obtained from Charles River Laboratories (Portage, WI) at 180 g and maintained in temperature- (23–25 C) and light-controlled rooms (14-h light, 10-h dark cycle, lights on 0700 h). Animals were fed standard Purina rodent chow (Purina Mills, LLC, St. Louis, MO) and had access to water *ad libitum*. The Animal Care and Use Committee of Northwestern University (Evanston, IL) approved the protocols, and animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals.

The *in vivo* experiments used a modified version of previously published steroid treatment protocol (13). Female Sprague Dawley rats were OVX at 1200 h on d 1. At 1200 h on d 6 and 7, they were given sc injections of 10 μg EB. At 1200 h on d 8, animals were decapitated and pituitary tissues snap-frozen. Another group of animals was OVX on d 1, treated with EB as described above, given sc injections of 500 μg of P_4 on d 9 at 1200 h, and killed at 1700 h on the same day. For tracking Y1R mRNA over the course of the estrous cycle, 9-wk-old rats in diestrus, proestrus, or estrus were killed at 1000, 1400, and 1900 h, respectively. Tissue levels of Y1R mRNA in the anterior pituitary were analyzed using competitive RT-PCR.

For experiments using tissue dispersion, rats at random stages of the estrous cycle were used to negate any influence of *in vivo* steroid levels on subsequent results. Animals were decapitated and anterior pituitaries immediately dispersed as described below.

Measurement of Y1R mRNA levels by competitive RT-PCR

For isolation of RNA from steroid-treated rats, tissues were homogenized in Tri Reagent using a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland). For isolation of RNA from dispersed cells in 12-well plates, Tri Reagent-LS was used. RNA was subsequently extracted by phenol/chloroform according to the Tri Reagent manufacturer's protocol (Molecular Research Center). All RNA samples were treated with RQ1 DNase I. Reverse transcription was performed using 2 μg of sample RNA in the presence of 50 pg competitor RNA. The competitor RNA used as a competitive standard was synthesized as previously described (13) using Y1R DNA encoding a 546-bp region spanning an intron of the Y1R gene. The corresponding region of Y1R mRNA was 436 bases in length. A standard curve for the competitive RT-PCR was constructed (13) and used to calculate sample Y1R mRNA levels, expressed in attomoles of Y1R mRNA per microgram of total sample RNA.

Tissue dispersion

Pituitary glands obtained from rats at random stages of the estrous cycle were placed in low-glucose DMEM containing 25 mM HEPES, 1% antibiotic/antibacterial, and 0.3% BSA. Pituitaries from 10 rats were pooled for each dispersion. Anterior pituitaries were rinsed in dissociation buffer (Hanks' balanced salt solution containing 25 mM HEPES) and diced into 1-mm^3 fragments on siliconized glass slides. The pituitary fragments were dispersed into single cells by incubation in dissociation buffer containing 4% (wt/vol) collagenase (type II), 4% (wt/vol) BSA, 2% (wt/vol) dextrose, and 80 U/ml DNase II for 2 h at 37 C in a 50-ml siliconized Bellco spinner flask (Bellco Glass, Inc., Vineland, NJ) with 95% oxygen added at the beginning and midpoint of the spin. Dispersed cells were centrifuged and resuspended in dissociation buffer containing 2.5% (wt/vol) pancreatin and incubated at 37 C in a spinner flask for 7 min. Fetal calf serum was added, and then the cells were again spun down and resuspended in either S-MEM with 0.5% BSA (for CELSEP) (Westcor, Logan, UT) or DMEM with 10% FBS (for plating). Cells were filtered through a 100- to 120-pore mesh nylon screen before being used immediately in the cell separation procedure or being plated in Matrigel-coated 12-well plates for later RNA extraction. This procedure yielded approximately 2–3.0 × 10^6 cells per pituitary.

Gonadotrope enrichment

Cells suspended in 30 ml of 0.5% BSA S-MEM at no more than 10^6 cells/ml were loaded into the chamber of a CELSEP unit gravity sedimentation device (Westcor) containing a gradient of 1.0–3.0% BSA in S-MEM. The protocol followed has been described previously (20) with the exception that cells were collected from the bottom of the gradient. Sedimentation rate using this procedure depends on differences in cell size and cell density (21).

Cell culture

For the Y1R expression studies, primary pituitary cells and cells from gonadotrope-enriched or -deprived pools were plated at 40 × 10^4 per well in Matrigel-coated 12-well plates in DMEM (4.5 g/liter glucose) containing 10% charcoal-stripped fetal calf serum and 100 pg/ml E_2 or vehicle control. After 24 h of incubation, RNA was extracted using Tri Reagent-LS as described.

For the secretion studies, cells from pool 3 (containing the majority of gonadotropes) or pool 2 (containing the majority of lactotropes) were resuspended in DMEM with 10% charcoal-treated FBS and plated on 48-well plates at 3.95 × 10^4 cells per well. They were then treated with 24 h of 50 pg/ml E_2 or vehicle alone, 4 h of 6.4 nM P_4 or vehicle, and/or 1 h of 500 nM BIBO3304 (a specific Y1R antagonist), or vehicle before the start of each experiment. Secretion was measured over 2 h of static culture in media containing the specified steroid hormones as well as GnRH (0.2 nM, unless otherwise noted), NPY (0.1 μM in LH studies, 0.2 μM in GH and PRL studies), BIBO3304TF (500 nM), or appropriate vehicle controls.

RIA

Serum LH was measured by RIA. The LH RIA used reagents provided by the National Institute of Diabetes and Digestive and Kidney

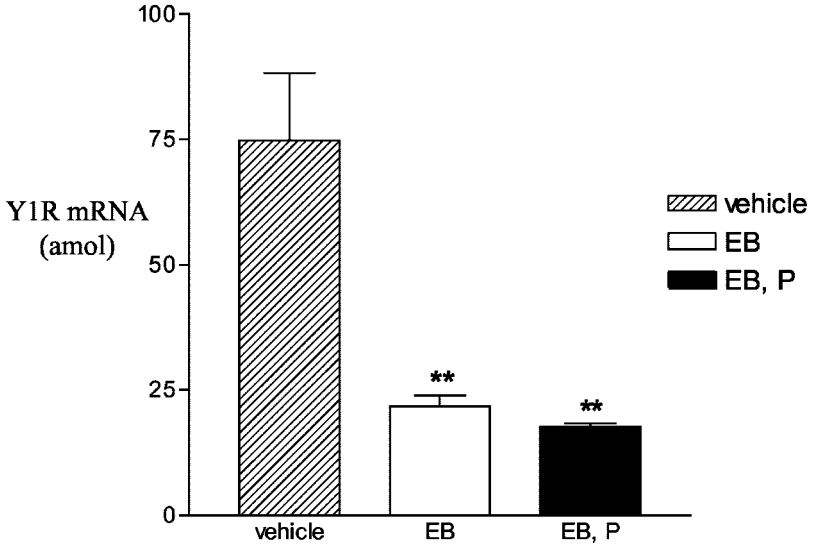


FIG. 1. Effect of EB and P_4 treatment on Y1R mRNA levels in rat pituitary tissue. Animals were OVX 6 d before the first injection. On d 1 and 2, animals received 10 μg of sc EB or oil at 1200 h. Animals treated with EB or vehicle alone were killed on the third day at 1200 h. On d 4, remaining animals received a 500-μg injection of P_4 at 1200 h and were killed at 1700 h. Levels of Y1R mRNA were significantly suppressed by EB. P_4 treatment did not further suppress pituitary Y1R mRNA levels (n = 4 per group). EB, E_2-treated; EB, P, E_2- and P_4-treated. **, Significantly different from vehicle control ($P < 0.01$).

Diseases, including the LH reference preparation (RP)-3. The LH assays had an interassay coefficient of variation of 12.8% and intraassay variation of 6.5%. The GH and PRL RIAs were also performed using reagents provided by the National Institute of Diabetes and Digestive and Kidney Diseases and had intraassay coefficients of variation of 8.8 and 8.1%, respectively.

Statistical analysis

Differences in levels of LH release or Y1R mRNA levels among different groups were assessed by one-way ANOVA followed by Newman-Keuls *post hoc* test. Student's *t* tests were used in two group comparisons. Differences were considered significant if $P < 0.05$.

Results

Pituitary Y1R mRNA levels in vivo

We investigated the impact of steroid hormone treatment on Y1R mRNA levels in the rat pituitary. OVX rats were given 10 μg EB injections for 2 d and killed on d 3. In contrast to previous results showing E_2-mediated up-regulation of Y1R mRNA in the hypothalamus (13), EB treatment substantially decreased the levels of Y1 receptor mRNA seen in pituitary tissue of OVX female rats (Fig. 1). A second group of animals was given the same EB treatment followed by 500 μg of P_4. P_4 administration caused no change in Y1R mRNA levels beyond that induced by E_2 (Fig. 1). When measured over the course of the estrous cycle, Y1R mRNA levels in the anterior pituitary were suppressed during proestrus, the point in the cycle at which E_2 levels are highest, and climbed again on the day of estrus (Fig. 2).

Y1R mRNA levels in gonadotrope-enriched and -depleted cell cultures

We next investigated the cell type in which this suppression occurs. As previously noted, the CELSEP system allows isolation of a population of cells that are 66–80% gonadotropes (pool 3). Pool 2 contains the majority of cells (52–67% of cells recovered), only 5% of which are LH positive, whereas pool 1 contains small cells and fibroblasts (23–32% of cells recovered) of which 0% are LH positive (20). Measurements of cellular LH content and basal secretion from these cells confirmed that this method was equally effective

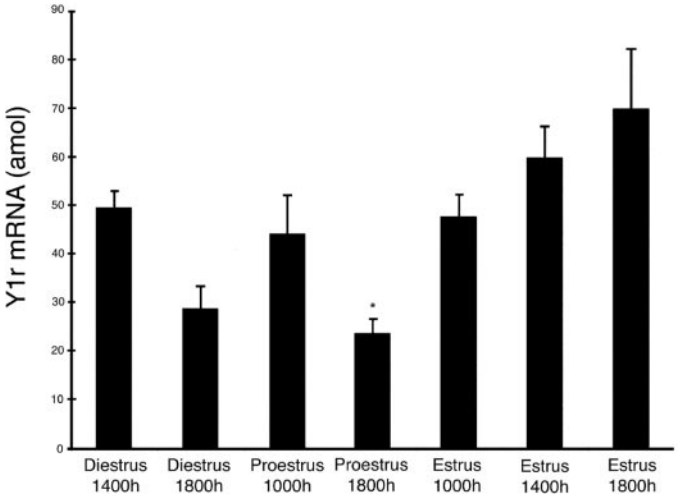


FIG. 2. Pituitary Y1 mRNA over the estrous cycle. Levels of Y1R mRNA are significantly depressed on the evening of proestrus. *, Significantly lower than diestrous 1400 and estrous groups ($P < 0.05$; n = 4–10 per group).

in our hands; Fig. 3 shows that basal LH release in pool 2 cultures showed no significant elevation above detectable limits, and GnRH-stimulated release was less than 7.5% of the GnRH-stimulated LH secretion in pool 3. On this basis, we therefore estimate that pool 3 cells include more than 92.5% of active LH-secreting gonadotropes. In addition, pool 2 releases the highest PRL levels, indicating concentration of lactotropes in pool 2 (Fig. 4).

Preliminary experiments entailing *in vitro* administration of E_2 for a 24-h period to dispersed primary anterior pituitary cells confirmed that the cells responded with a decrease in Y1r mRNA, indicating that these cells remain viable and respond in an appropriate manner to E_2 treatment (Fig. 5). We then used a gonadotrope-enrichment technique to determine whether the suppression inY1R mRNA was occurring in gonadotropes. We tested the response of the gonadotrope-enriched (pool 3) *vs.* gonadotrope-depleted (pool 1 and 2) cell populations to 24 h of incubation with 100 pg/ml

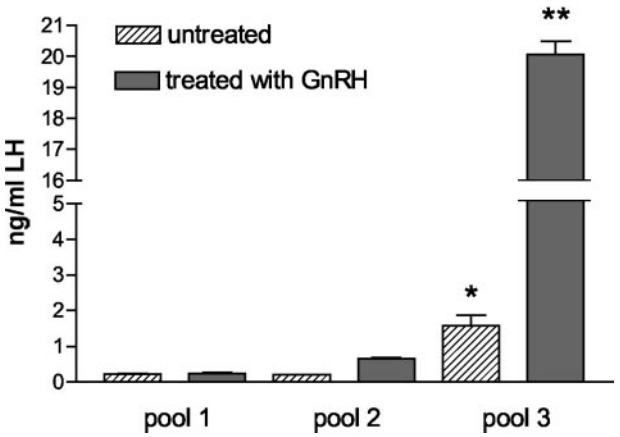


FIG. 3. Basal and GnRH-induced LH release from each of the three cell populations isolated by unit gravity sedimentation. *, Significantly different from pools 1 and 2 untreated and treated controls ($P < 0.05$); **, significantly different from all other groups ($P < 0.01$; n = 8–12 wells per group).

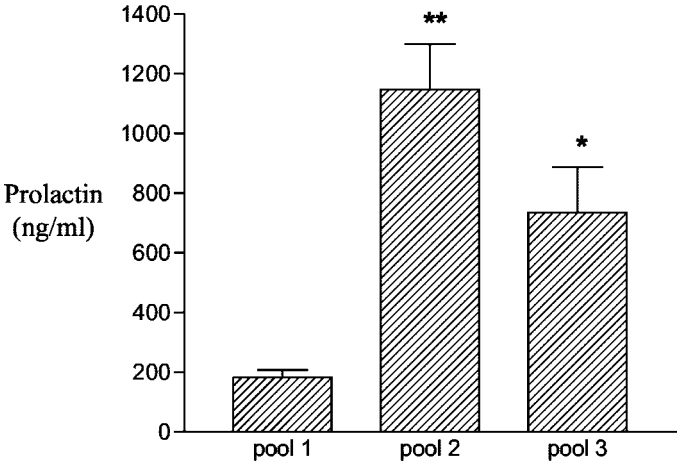


FIG. 4. Basal PRL release from each of the three cell populations isolated by unit gravity sedimentation. *, Significantly different from pool 1 ($P < 0.05$); **, significantly different from all other groups ($P < 0.01$; n = 3–4 samples per group).

E_2 treatment. Pool 2 cells displayed a suppression of Y1R mRNA levels, whereas pool 3 showed a significant elevation of Y1R mRNA in response to E_2 (Fig. 6). We suspect that the relative paucity of gonadotropes in the anterior pituitary masks the stimulatory effects of E_2 on these cells when measured at the tissue level. In addition, E_2 may suppress Y1R levels in cell types not isolated by this sedimentation technique.

LH release by gonadotrope-enriched cells

Because an increase in Y1R expression might alter LH secretion by the gonadotrope, we sought to establish whether NPY directly affects gonadotrope LH secretion. As previously mentioned, the ability of NPY to augment GnRH-induced LH release *in vivo* requires a proestrous (high estrogen) hormonal environment. Thus, we first tested the responsiveness of gonadotrope-enriched cells cultured without steroids. After 24 h of equilibration in steroid-free media, secretion was measured over 2 h of static culture with 0.2 nM GnRH. The cells responded to GnRH treatment with a significant increase in LH release, but 0.1 μM NPY on its own or in combination with GnRH did not increase secretion (Fig. 7A). In contrast, when the cells were pretreated and cultured with E_2 (50 pg/ml), NPY augmented the LH release induced by GnRH (Fig. 7B). The addition of BIBO3304 (500 nM), a Y1R-specific antagonist, reduced LH levels to the levels produced by GnRH alone.

Finally, when pretreated in the presence of P_4 alone (6.4 nM) for 4 h followed by measurement over a 2-h span, NPY significantly decreased rather than increased GnRH-induced LH release. The addition of BIBO3304 (500 nM) did not fully restore LH levels to those induced by GnRH alone (Fig. 8).

PRL and GH release by gonadotrope-depleted cells

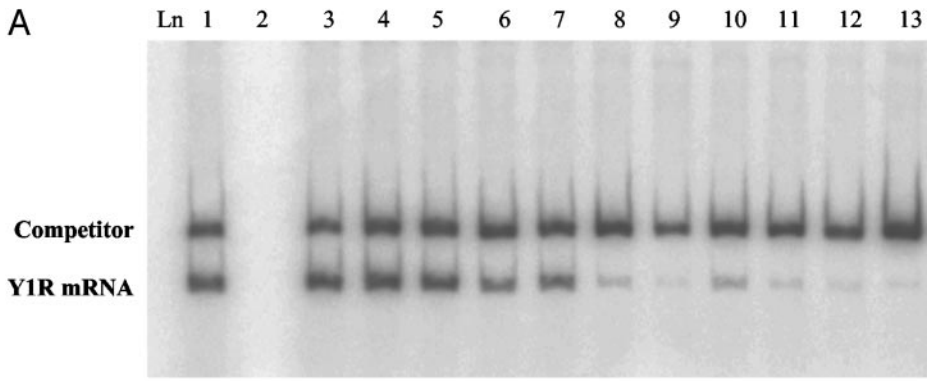
Finally, we pursued the possible effects of the suppression of Y1R mRNA levels on secretory activity in gonadotrope-depleted pool 2. Because somatotropes and lactotropes predominate in the pituitary, we focused on whether GH or PRL release was altered in response to E_2. Basal GH release increased in response to E_2 treatment (50 pg/ml) over the 2-h culture period only in the absence of NPY (Fig. 9A). In contrast, 0.2 μM NPY suppressed PRL secretion in an E_2-free environment, and NPY failed to affect PRL secretion in the presence of E_2 (Fig. 9B). As expected, the 2-h incubation period was too short a period to see a direct influence of E_2 treatment on PRL secretion (22).

We investigated these effects further in unseparated dispersed rat pituitary cells. In the absence of E_2, we again found that NPY suppressed PRL release; however, this suppression was not altered by the addition of 500 nM BIBO3304 (Fig. 10).

Discussion

These experiments establish that gonadotrope-enriched cell populations show a significant elevation of Y1R mRNA in response to E_2 exposure. In addition, *in vitro* secretion studies with these cells suggest that the gonadotrope Y1R mediates NPY's actions on pituitary LH release in the presence of E_2. Our results provide evidence that NPY requires no intermediate cell type to exert its actions on pituitary LH release. In addition, these results shed light on the preparation of the pituitary for the preovulatory LH surge. Although estrogen-positive feedback at the hypothalamus is known to increase GnRH release at the time of the surge, it has long been recognized that a dramatic increase in pituitary responsiveness to GnRH input on proestrus represents an equal or greater factor in initiation of the LH surge. These experiments suggest one mechanism by which estrogen heightens pituitary responsiveness to GnRH on the day of proestrus. By up-regulating Y1R expression in gonadotropes, estrogen may trigger NPY's amplifying effects on GnRH-induced LH release.

Because E_2 treatment induced a major suppression of Y1R mRNA levels in whole pituitaries, we also sought to identify the cell type in which E_2-induced suppression of Y1R mRNA occurs. Immunohistochemical studies have found that the intact female pituitary contains approximately 27% somatotrope, 25% lactotrope, 12% FSH-gonadotrope, 9% LH-gonadotrope, 8% ACTH, 4% TSH-containing cells (23), and 5–10%



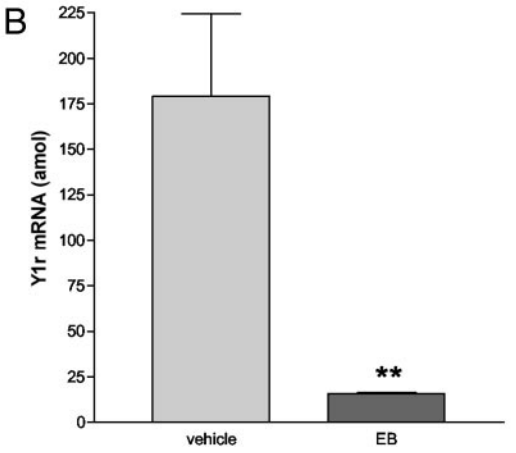


FIG. 5. Y1R levels in dispersed anterior pituitary cells with and without *in vitro* E_2 treatment (100 pg/ml). A, PAGE of RT-PCR-generated products visualized by phosphoimage analysis. *Top bands* (546 bp) correspond to added competitor RNA, whereas *bottom bands* (436 bp) correspond to sample mRNA levels. Lanes 1 and 3–6 were E_2 treated; lanes 7–12 were vehicle treated; and lane 2 was left empty to help orient the gel after visualization. Band ratios were taken, and quantitative mRNA levels, calculated using standard curve, were graphed (B). **, Significantly different from vehicle control ($P < 0.01$).

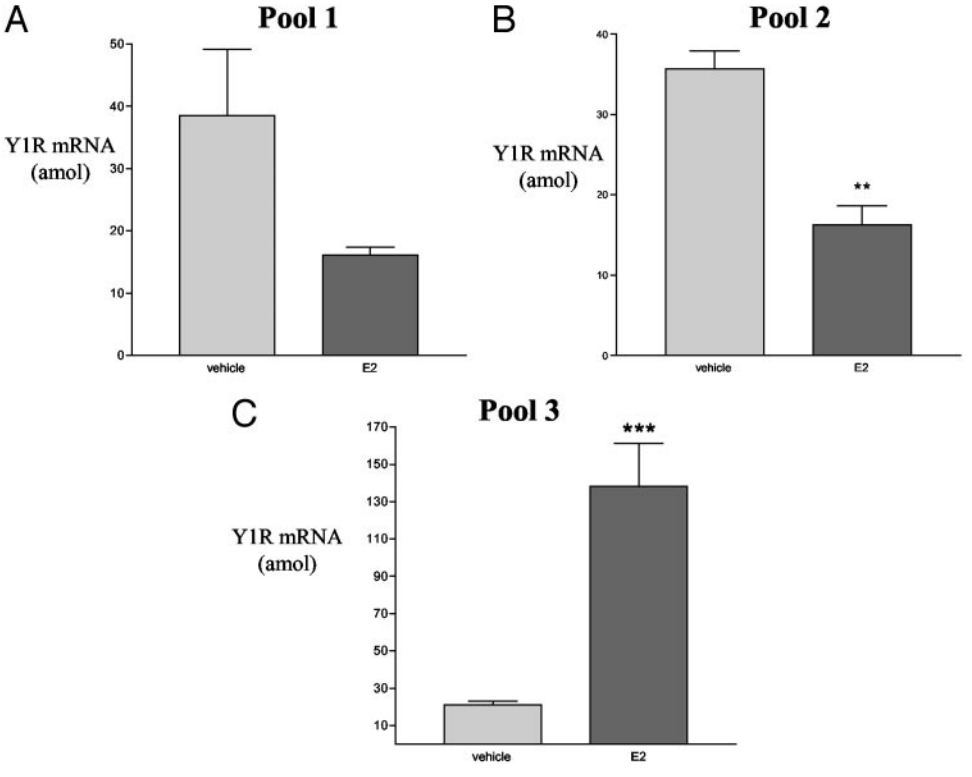


FIG. 6. Effect of 100 pg/ml *in vitro* E_2 treatment on Y1R mRNA levels in dispersed pituitary cells after separation. E_2 significantly suppressed levels in pool 2 cells ($P < 0.01$) and increased levels in pool 3 ($P < 0.001$; n = 4 samples per group).

folliculostellate cells, with the remainder consisting of non-endocrine cells such as connective tissue, immune cells, and fenestrated capillaries (24). In the short-term OVX rat pituitary, approximately 47% of lactotropes, 35% of corticotropen, 26% of gonadotropes, and 35% of folliculostellate cells express ERα, whereas 27% of lactotropes, 25% of cortico-

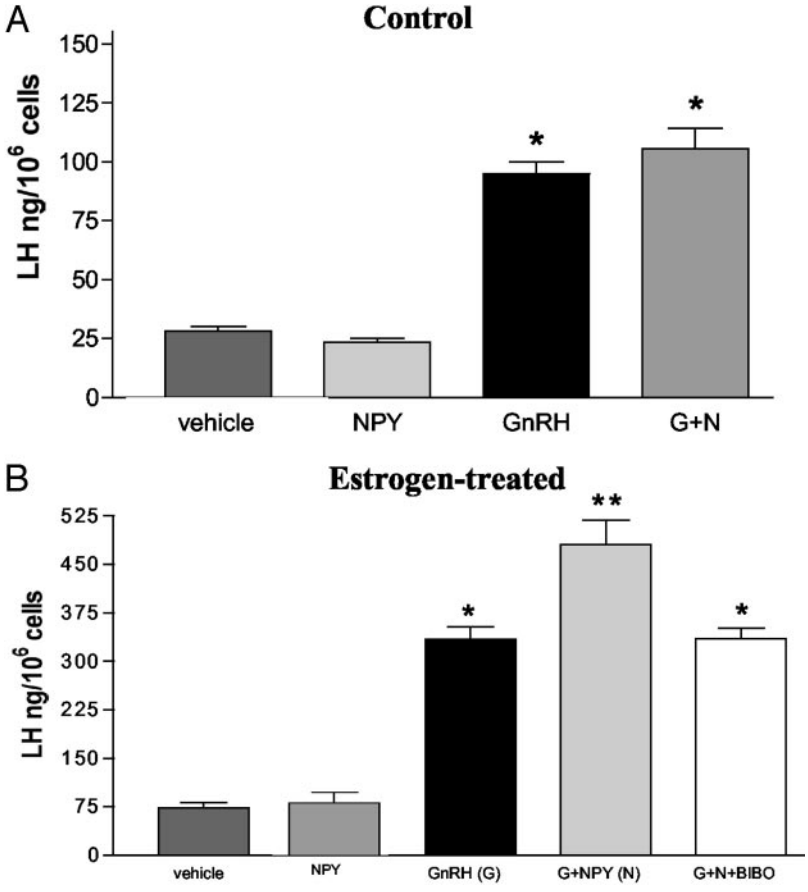


FIG. 7. LH release from pool 3 in the absence and presence of E_2 (50 pg/ml). In panel A, steroid-free media were used, whereas in panel B, E_2 was added to the media. *, Significantly elevated above vehicle ($P < 0.05$); **, significantly elevated above all other groups ($P < 0.01$; n = 15–18 wells per group). G, GnRH (0.2 mM); N, NPY (0.1 μM); BIBO, BIBO3304 (500 nM; Y1 receptor-specific antagonist).

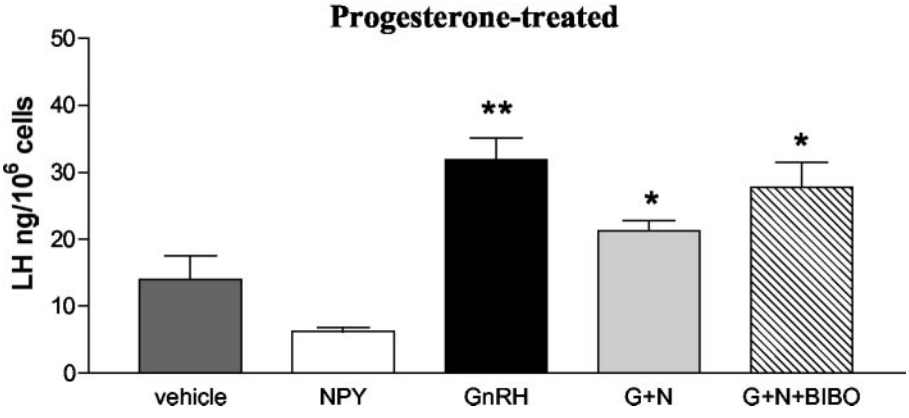


FIG. 8. LH release by pool 3 in the presence of P_4 (6.4 nM). *, Significantly elevated above vehicle ($P < 0.05$); **, significantly elevated above all other groups ($P < 0.01$; n = 15–18 wells per group). G, GnRH (0.2 mM); N, NPY (0.1 μM); BIBO, BIBO3304 (500 nM; Y1 receptor-specific antagonist).

tropes, 17% of gonadotropes, and 30% of folliculostellate cells express ERβ (25). Because only 8–10% of anterior pituitary cells coexpress both ERα and ERβ (25), many pituitary cells are potentially E_2 responsive. We found that in a lactotrope-rich population of anterior pituitary cells, E_2 treatment suppressed Y1R mRNA levels. These results demonstrate the existence of multiple pathways leading to Y1R mRNA expression in different NPY target tissues and even among different cell types. Indeed, Musso *et al*. (14) have found that E_2 induces transcription of a luciferase-fused Y1R promoter in NG108-15 cells but not in SK-N-BE cells, suggesting that cell type-specific factors are required for ERα activity. However, because Y1R blockade failed to reverse NPY's effects on PRL secretion, the functional significance of any such effect remains unclear. Likewise, because these separation studies did not result in pure cell type populations, we cannot exclude the possibility that this down-regulation occurs in another cell type found in pool 2.

NPY consistently suppresses LH release when administered to OVX animals or to intact animals on a chronic basis (26, 27). However, it is uncertain whether these suppressive actions are exerted on hypothalamic or pituitary targets. Isolated metestrous pituitaries have been shown to exhibit either a suppression of LH release (28) or no response to NPY treatment (9). Our current experiments demonstrate a suppression of LH release only when P_4 is present in the absence of E_2. Even under these conditions, NPY was not able to prevent a significant increase in LH as a result of GnRH

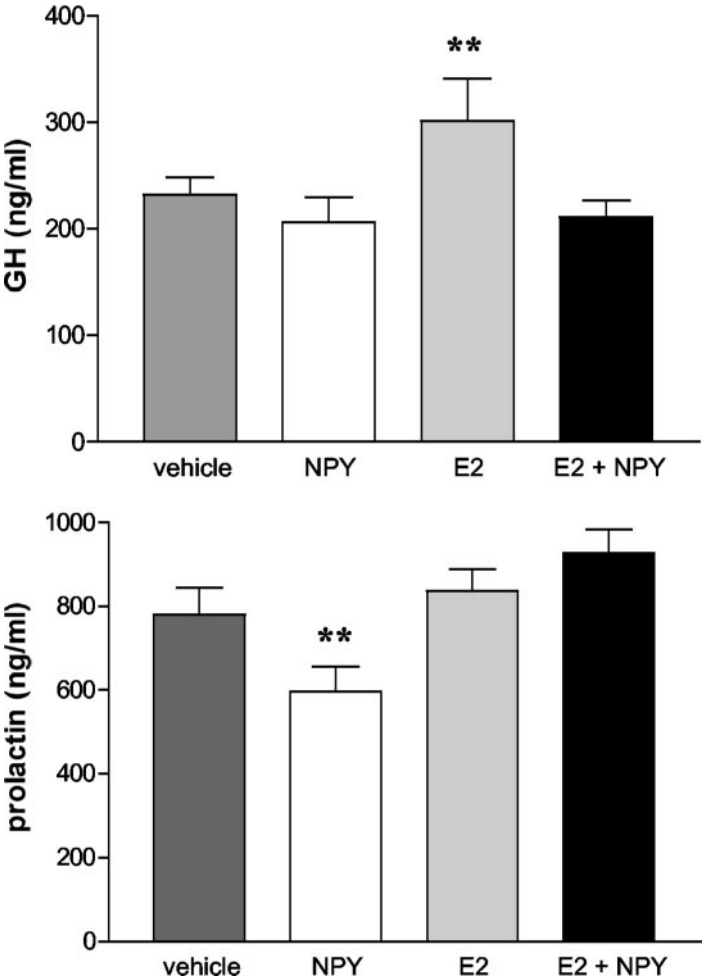


FIG. 9. GH and PRL levels after 2 h E_2 (50 pg/ml) and NPY (0.2 μM) treatment of pool 2 cells. In panel A, E_2 but not NPY significantly elevated GH release, whereas in panel B NPY suppressed PRL release in the absence of E_2. **, Significantly different from vehicle ($P < 0.01$; n = 20 wells per group).

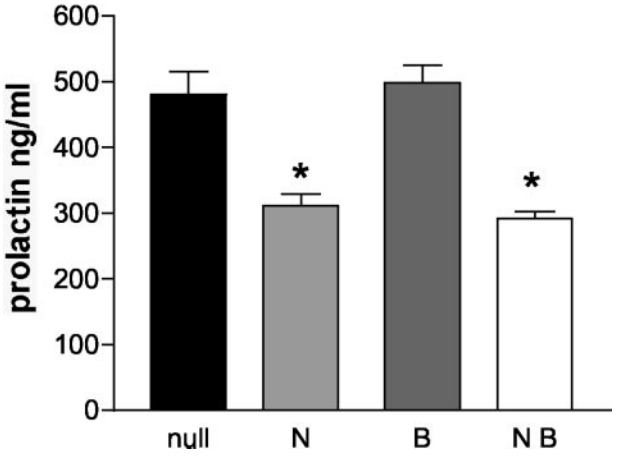


FIG. 10. Dispersed primary pituitary cells were treated with NPY (0.2 μM). A 2-h incubation with NPY significantly reduced PRL secretion over the 2-h incubation period in the presence and absence of the Y1R antagonist BIBO3304. No steroids were present in the media (n = 32 all groups except BIBO alone, in which n = 13). N, NPY; B, BIBO3304 (Y1 receptor antagonist); NB, NPY + BIBO3304. ***, Significantly different from vehicle ($P < 0.001$).

administration. These data suggest that the locus of LH suppression exerted by NPY may primarily reside in the hypothalamus, with a minor P_4-dependent effect occurring at the pituitary level.

Our observation that NPY reduces GnRH-induced LH release in our gonadotrope-enriched cells treated with P_4 alone raises questions in light of previous research. P_4 is present at elevated levels during the LH surge in the rat and augments surge size (30, 31). In addition, acute P_4 exposure (3–6 h) of pituitary cells *in vitro* increases responsiveness to GnRH (32–34). However, in these cases, the pituitary cells had also been exposed to E_2. E_2 increases P_4 receptor mRNA and protein levels in the pituitary, whereas P_4 down-regulates its own receptor (35–39). In addition, E_2-inducible PR in the pituitary can be activated in a ligand-independent manner, giving rise to enhanced GnRH-stimulated LH secretion (40). Our cells received no E_2 priming for the 24 h before the experiment or during the testing period. These facts suggest that the facilitative effects of NPY may require the presence of P_4 receptors, and in their absence, the inhibitory effects of NPY on LH release become apparent (28). These results are consistent with the finding that sc injected RU486 prevents NPY facilitation of LH surges in proestrous rats (7). Further experimentation is required to test this conjecture.

The results of the present studies confirm previous findings that NPY suppresses PRL secretion from rat anterior pituitary cells (17) and agree with *in vivo* data showing suppression of PRL in response to intracerebroventricular NPY administration in males (41) and during lactation (42). Past research has found that cells cultured for 3 d in media without added E_2 showed suppressed PRL levels, whether the cells came from lactating animals or from OVX steroid-replaced rats (17). Similarly, this study shows that GH/PRL-enriched cells from randomly cycling rats cultured without E_2 show NPY-suppressed PRL levels, whereas cells cultured in media with added E_2 showed no PRL suppression. However, in the presence of all pituitary cell types, lactotropes do show an NPY-induced suppression of PRL in the presence of E_2 that Y1R blockade reverses. Thus, NPY may act on neighboring cells types through Y1Rs to cause the release of paracrine factors that suppress PRL release.

The present results illustrate another mechanism by which E_2 acts to prepare the hypothalamic-pituitary axis on multiple levels for the preovulatory LH surge. We previously showed that E_2 increases NPY Y1R expression in the hypothalamus on the proestrus afternoon in a manner that may permit NPY to facilitate GnRH release. We have now shown that E_2 acts specifically on gonadotropes to up-regulate Y1R expression, and in the absence of E_2, NPY cannot augment GnRH-induced LH release from these cells. Such cell-specific steroid-mediated alterations in Y1R expression may result from different ERs, corepressors, or coactivators present in different cell types or from activation of different intracellular pathways. For instance, NPY has been found to reduce Ca^{2+} entry through voltage-dependent Ca^{2+} channels in lactotropes (17) and to facilitate Ca^{2+} influx in gonadotropes (43). The precise mechanisms responsible for the targeted effects on NPY receptor-mediated responses examined in these studies will require further investigation.

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References

1. **Woller MJ, Terasawa E** 1992 Estradiol enhances the action of neuropeptide Y on *in vivo* luteinizing hormone-releasing hormone release in the ovariectomized rhesus monkey. Neuroendocrinology 56:921–925
2. **Woller MJ, McDonald JK, Reboussin DM, Terasawa E** 1992 Neuropeptide Y is a neuromodulator of pulsatile luteinizing hormone-releasing hormone release in the gonadectomized rhesus monkey. Endocrinology 130:2333–2342
3. **Watanobe H, Takebe K** 1992 Evidence that neuropeptide Y secretion in the median eminence increases prior to the luteinizing hormone surge in ovariectomized steroid-primed rats: estimation by push-pull perfusion. Neurosci Lett 146:57–59
4. **Sutton SW, Toyama TT, Otto S, Plotsky PM** 1988 Evidence that neuropeptide Y (NPY) released into the hypophysial-portal circulation participates in priming gonadotropes to the effects of gonadotropin releasing hormone (GnRH). Endocrinology 123:1208–1210
5. **Xu M, Hill JW, Levine JE** 2000 Attenuation of luteinizing hormone surges in neuropeptide Y knockout mice. Neuroendocrinology 72:263–271
6. **Bauer-Dantoin AC, McDonald JK, Levine JE** 1991 Neuropeptide Y potentiates luteinizing hormone (LH)-releasing hormone-stimulated LH surges in pentobarbital-blocked proestrous rats. Endocrinology 129:402–408
7. **Bauer-Dantoin AC, Tabesh B, Norgle JR, Levine JE** 1993 RU486 administration blocks neuropeptide Y potentiation of luteinizing hormone (LH)-releasing hormone-induced LH surges in proestrous rats. Endocrinology 133:2418–2423
8. **Besecke LM, Levine JE** 1994 Acute increase in responsiveness of luteinizing hormone (LH)-releasing hormone nerve terminals to neuropeptide-Y stimulation before the preovulatory LH surge. Endocrinology 135:63–66
9. **Leupen SM, Besecke LM, Levine JE** 1997 Neuropeptide Y Y1-receptor stimulation is required for physiological amplification of preovulatory luteinizing hormone surges. Endocrinology 138:2735–2739
10. **Bauer-Dantoin AC, McDonald JK, Levine JE** 1992 Neuropeptide Y potentiates luteinizing hormone (LH)-releasing hormone-induced LH secretion only under conditions leading to preovulatory LH surges. Endocrinology 131:2946–2952
11. **Bauer-Dantoin AC, Knox KL, Schwartz NB, Levine JE** 1993 Estrous cycle stage-dependent effects of neuropeptide-Y on luteinizing hormone (LH)-releasing hormone-stimulated LH and follicle-stimulating hormone secretion from anterior pituitary fragments *in vitro*. Endocrinology 133:2413–2417
12. **Rudolf K, Eberlein W, Engel W, Beck-Sickinger A, Wittneben H, Doods H** 1997 BIBP3226, a potent and selective neuropeptide Y Y1-receptor antagonist. Structure-activity studies and localization of the human Y1 receptor binding. In: Grundemar L, Bloom S, eds. Neuropeptide Y and Drug Development. San Diego: Academic Press; 175–190
13. **Xu M, Urban JH, Hill JW, Levine JE** 2000 Regulation of hypothalamic neuropeptide Y Y1 receptor gene expression during the estrous cycle: role of progesterone receptors. Endocrinology 141:3319–3327
14. **Musso R, Maggi A, Eva C** 2000 17 β-estradiol stimulates mouse neuropeptide Y-Y(1) receptor gene transcription by binding to estrogen receptor α in neuroblastoma cells. Neuroendocrinology 72:360–367
15. **Hsueh YC, Cheng SM, Pan JT** 2002 Fasting stimulates tuberoinfundibular dopaminergic neuronal activity and inhibits prolactin secretion in oestrogen-primed ovariectomized rats: involvement of orexin A and neuropeptide Y. J Neuroendocrinol 14:745–752
16. **Silveira NA, Franci CR** 1999 Antisense mRNA for NPY-Y1 receptor in the medial preoptic area increases prolactin secretion. Braz J Med Biol Res 32:1161–1165
17. **Wang J, Ciofi P, Crowley WR** 1996 Neuropeptide Y suppresses prolactin secretion from rat anterior pituitary cells: evidence for interactions with dopamine through inhibitory coupling to calcium entry. Endocrinology 137:587–594
18. **Garcia de Yebenes E, Li S, Fournier A, St-Pierre S, Pelletier G** 1995 Involvement of the Y2 receptor subtype in the regulation of prolactin gene expression by neuropeptide Y in the male rat. Neurosci Lett 190:77–80
19. **Chao CC, Scribner KA, Dixon JE, Malven PV** 1987 Failure of neuropeptide Y to modulate the release of LH and prolactin by cultured bovine pituitary cells. Domest Anim Endocrinol 4:309–314
20. **Turgeon JL, Waring DW** 1994 Activation of the progesterone receptor by the gonadotropin-releasing hormone self-priming signaling pathway. Mol Endocrinol 8:860–869
21. **Hymer WC, Hatfield JM** 1983 Separation of cells from the rat anterior pituitary gland. Methods Enzymol 103:257–287
22. **Lagace L, Labrie F, Antakly T, Pelletier G** 1981 Sensitivity of rat adenohypophyseal cells to estradiol and LHRH during long-term culture. Am J Physiol 240:E602–E608
23. **Ibrahim SN, Moussa SM, Childs GV** 1986 Morphometric studies of rat anterior pituitary cells after gonadectomy: correlation of changes in gonadotropes with the serum levels of gonadotropins. Endocrinology 119:629–637
24. **Vila-Porcile E** 1972 [The network of the folliculo-stellate cells and the follicles of the adenohypophysis in the rat (pars distalis)]. Z Zellforsch Mikrosk Anat 129:328–369 (French)
25. **Mitchner NA, Garlick C, Ben-Jonathan N** 1998 Cellular distribution and gene regulation of estrogen receptors α and β in the rat pituitary gland. Endocrinology 139:3976–3983
26. **Sahu A, Crowley WR, Tatemoto K, Balasubramaniam A, Kalra SP** 1987 Effects of neuropeptide Y, NPY analog (norleucine4-NPY), galanin and neuropeptide K on LH release in ovariectomized (ovx) and ovx estrogen, progesterone-treated rats. Peptides 8:921–926
27. **Catzeflis C, Pierroz DD, Rohner-Jeanrenaud F, Rivier JE, Sizonenko PC, Aubert ML** 1993 Neuropeptide Y administered chronically into the lateral ventricle profoundly inhibits both the gonadotropic and the somatotropic axis in intact adult female rats. Endocrinology 132:224–234
28. **Knox KL, Bauer-Dantoin AC, Levine JE, Schwartz NB** 1995 Unmasking of neuropeptide-Y inhibitory effects on *in vitro* gonadotropin secretion from pituitaries of metestrous, but not proestrous, rats. Endocrinology 136:187–194
29. Deleted in proof
30. **Chang RJ, Jaffe RB** 1978 Progesterone effects on gonadotropin release in women pretreated with estradiol. J Clin Endocrinol Metab 47:119–125
31. **Odell WD, Swerdloff RS** 1968 Progestogen-induced luteinizing and follicle-stimulating hormone surge in postmenopausal women: a simulated ovulatory peak. Proc Natl Acad Sci USA 61:529–536
32. **Lagace L, Massicotte J, Labrie F** 1980 Acute stimulatory effects of progesterone on luteinizing hormone and follicle-stimulating hormone release in rat anterior pituitary cells in culture. Endocrinology 106:684–689
33. **Ortmann O, Wiese H, Knuppen R, Emons G** 1989 Acute facilitatory action of progesterone on gonadotropin secretion of perifused rat pituitary cells. Acta Endocrinol (Copenh) 121:426–434
34. **Krey LC, Kamel F** 1990 Progesterone modulation of gonadotropin secretion by dispersed rat pituitary cells in culture. I. Basal and gonadotropin-releasing hormone-stimulated luteinizing hormone release. Mol Cell Endocrinol 68:85–94
35. **Evans RW, Sholiton LJ, Leavitt WW** 1978 Progesterone receptor in the rat anterior pituitary: effect of estrogen priming and adrenalectomy. Steroids 31:69–81
36. **Moguilewsky M, Raynaud JP** 1979 Estrogen-sensitive progestin-binding sites in the female rat brain and pituitary. Brain Res 164:165–175
37. **Turgeon JL, Van Patten SM, Shyamala G, Waring DW** 1999 Steroid regulation of progesterone receptor expression in cultured rat gonadotropes. Endocrinology 140:2318–2325
38. **Turgeon JL, Waring DW** 2000 Progesterone regulation of the progesterone receptor in rat gonadotropes. Endocrinology 141:3422–3429
39. **Szabo M, Kilen SM, Nho SJ, Schwartz NB** 2000 Progesterone receptor A and B messenger ribonucleic acid levels in the anterior pituitary of rats are regulated by estrogen. Biol Reprod 62:95–102
40. **Bellido C, Aguilar R, Garrido-Gracia JC, Sanchez-Criado JE** 2002 Effects of progesterone (P) and antiprogestin RU486 on LH and FSH release by incubated pituitaries from rats treated with the SERM LY11701 8-HCl and/or recombinant human FSH. J Endocrinol Invest 25:702–708
41. **Reznikov AG, McCann SM** 1993 Effects of neuropeptide Y on gonadotropin and prolactin release in normal, castrated or flutamide-treated male rats. Neuroendocrinology 57:1148–1154
42. **Toufexis DJ, Yorozu S, Woodside B** 2002 Y1 receptor activation is involved in the effect of exogenous neuropeptide Y on pup growth and the early termination of lactational diestrus in the postpartum rat. J Neuroendocrinol 14:354–360
43. **Crowley WR, Shah GV, Carroll BL, Kennedy D, Dockter ME, Kalra SP** 1990 Neuropeptide-Y enhances luteinizing hormone (LH)-releasing hormone-induced LH release and elevations in cytosolic Ca^{2+} in rat anterior pituitary cells: evidence for involvement of extracellular Ca^{2+} influx through voltage-sensitive channels. Endocrinology 127:1487–1494

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