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

JENNIFER W. HILL, JANICE H. URBAN, MING XU, AND JON E. LEVINE

Department of Neurobiology and Physiology (J.W.H., J.H.U., M.X., J.E.L.), Northwestern University, Evanston, Illinois 60208; and Department of Physiology and Biophysics (J.H.U.), Finch University of Health Sciences/The Chicago Medical School, North Chicago, Illinois 60064

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 (E2) treatment likewise suppressed Y1R expression. In contrast, E2 elevated Y1R mRNA in gonadotrope-enriched cell populations, indicating that estrogen regulates Y1R mRNA expression differently in gonadotrope vs. other pituitary cell types. After exposure to E2, 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 E2 can directly up-regulate gonadotrope responsiveness to NPY and suggest that this action is mediated at least in part by E2’s ability to stimulate Y1R gene expression in gonadotropes. Our observations are consistent with the idea that this regulatory mechanism represents a component of E2’s positive feedback actions in pituitary gonadotropes. The biological importance of E2’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, proestrus 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 proestrus 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, proestrus 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 (E2) levels are high, and E2 administration replicates this gain (13). In addition, Musso et al. (14) found that E2 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; E2, 17β-estradiol; EB, E2 benzoate; ERα, estrogen receptor α; ERβ, estrogen receptor β; FBS, fetal bovine serum; NPY, neuropeptide Y; OVX, ovariectomized; P4, progesterone; PRL, prolactin; S-MEM, suspension MEM; Y1R, neuropeptide Y1 receptor.

Endocrinology is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.
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 hypophyseal 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 (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.

**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 Belco spinner flask (Belco 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 2–3×10\(^5\) 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 (10) 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\(^3\) 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 mM 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 Diseases.
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 of EB injections for 2 d and killed on d 3. In contrast to previous results showing E2-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 P4 administration caused no change in Y1R mRNA levels beyond that induced by E2 (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 E2 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 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 E2 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 E2 treatment (Fig. 5). We then used a gonadotrope-enrichment technique to determine whether the suppression in Y1R 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
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 E2 (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 P4 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 E2. Basal GH release increased in response to E2 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 E2-free environment, and NPY failed to affect PRL secretion in the presence of E2 (Fig. 9B). As expected, the 2-h incubation period was too short a period to see a direct influence of E2 treatment on PRL secretion (22).

We investigated these effects further in unseparated dispersed rat pituitary cells. In the absence of E2, 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 E2 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 E2. 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 E2 treatment induced a major suppression of Y1R mRNA levels in whole pituitaries, we also sought to identify the cell type in which E2-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%
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 corticotropes, 26% of gonadotropes, and 35% of folliculostellate cells express ERα, whereas 27% of lactotropes, 25% of corticotropes, 26% of gonadotropes, and 35% of folliculostellate cells express ERβ. **Significantly different from vehicle control (P < 0.01).**
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 E2 responsive. We found that in a lactotroperich population of anterior pituitary cells, E2 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 E2 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 P4 is present in the absence of E2. Even under these conditions, NPY was not able to prevent a significant increase in LH as a result of GnRH
administration. These data suggest that the locus of LH suppression exerted by NPY may primarily reside in the hypothalamus, with a minor P₄-dependent effect occurring at the pituitary level.

Our observation that NPY reduces GnRH-induced LH release in our gonadotrope-enriched cells treated with P₄ alone raises questions in light of previous research. P₄ is present at elevated levels during the LH surge in the rat and augments surge size (30, 31). In addition, acute P₄ 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 E₂. E₂ increases P₄ receptor mRNA and protein levels in the pituitary, whereas P₄ down-regulates its own receptor (35–39). In addition, E₂-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 E₂ 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 P₄ 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 E₂ 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 E₂ 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 E₂ 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 E₂ acts to prepare the hypothalamic-pituitary axis on multiple levels for the preovulatory LH surge. We previously showed that E₂ 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 E₂ acts specifically on gonadotropes to up-regulate Y1R expression, and in the absence of E₂, 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 Ca²⁺ entry through voltage-dependent Ca²⁺ channels in lactotropes (17) and to facilitate Ca²⁺ 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.
References

1. Woller MJ, Terasawa E 1992 Estradiol enhances the action of neuropeptide Y on in vivo luteinizing hormone-releasing hormone release in the ovario-
mized rhesus monkey. Neuroendocrinology 56:921–925

2. Woller MJ, McDonald JK, Reussinn DM, Terasawa E 1992 Neuropeptide Y is a neuropepduer of pulsatile luteinizing hormone-releasing hormone re-
lease in the gonadotropinized 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 ovario-
ekotomized steroid-primed rats: estimation by push-pull perfusion. Neurosci
Lett 146:57–59

4. Sutton SW, Toyama TT, Otto S, Plotsky PM 1992 Evidence that neuropeptide Y (NPY) injected into the hypophyseal-portal circulation participates in prim-
ing gonadotropes to the effects of gonadotropin releasing hormone (GnRH).
Endocrinology 123:1208–1210


6. Bauer-Dantoin AC, McDonald JK, Levine JE 1991 Neuropeptide Y potentiates luteinizing hormone (LH)-releasing hormone-stimulated LH surges in pento-
barbital-blocked proestrus rats. Endocrinology 129:402–408

7. Bauer-Dantoin AC, Tabesh B, Norgle JR, Levine JE 1993 RU486 administra-
tion blocks neuropeptide Y potentiation of luteinizing hormone (LH)-releasing
hormone-induced LH surges in proestrus rats. Endocrinology 133:2418–2423

hormone (LH)-releasing hormone nerve terminals to neuropeptide-Y stimu-
lization before the preovulatory LH surge. Endocrinology 135:63–66

9. Leupen SM, Besecke LM, Levine JE 1997 Neuropeptide Y Y1-receptor stimu-
lation 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 un-
der conditions leading to preovulatory LH surges. Endocrinology 131:2946–
2952

releasing hormone-stimulated LH and follicle-stimulating hormone secretion in female pituitary fragments in vitro. Endocrinology 133:2413–2417

Gründemar L, Bloom S, eds. Neuropeptide Y and Drug Development. San
Diego: Academic Press; 175–190

peptide Y Y1 receptor gene expression during the estrous cycle: role of progesterone receptors. Endocrinology 141:3319–3327

Y(Y1) receptor gene transcription by binding to estrogen receptor α in neo-
br reblastoma cells. Neuroendocrinology 72:360–367

15. Hseuh YC, Cheng SM, Pan JT 2002 Fasting stimulates tuberoinfundibular
dopaminergic neuronal activity and inhibits prolactin secretion in oestro-
gen-primed ovarioctomized rats: effects of oremexin A and neuropeptide Y J Neuroendocrinol 14:745–752

16. Silveira NA, Franci CR 1999 Antisense mRNA for NPY-Y1 receptor in the medi-
ul preoptic area increases prolactin secretion. Braz J Med Biol Res 32:
1161–1165

17. Wang J, Cifio P, Crowley WR 1996 Neuropeptide Y suppresses prolactin secretion from rat anterior pituitary cells: evidence for interactions with do-
pamine 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 Involv-
ment of the Y2 receptor subtype in the regulation of prolactin gene expression