

Regulation of Hypothalamic Neuropeptide Y Y1 Receptor Gene Expression during the Estrous Cycle: Role of Progesterone Receptors*

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ABSTRACT

Neuropeptide Y (NPY) stimulates the release of GnRH in an estrogen (E_2)-dependent manner, which is important in generating preovulatory GnRH surges. We tested the hypothesis that E_2 up-regulates NPY's actions by stimulating NPY Y1 receptor (Y1r) gene expression through a mechanism mediated by E_2 's ability to induce progesterone (P) receptors (PRs). In initial experiments, a specific Y1r antagonist BIBP3226 was used to confirm the involvement of Y1r in the stimulatory effects of NPY on *in vivo* GnRH release. Hypothalamic Y1r messenger RNA (mRNA) levels were then measured using competitive RT-PCR and were found to be significantly increased at 1000, 1200, and 1400 h on proestrus compared with other times of the day or cycle stage. Ovariectomy eliminated these increases, and E_2 treatment restored them. Additional P treatment produced even larger increases in Y1r mRNA levels. To assess the role of PRs in stimulating

Y1r expression, proestrous rats were treated with PR antagonist or oil vehicle and killed at 1200 h. Treatment with PR antagonist completely blocked the proestrous rise in Y1r gene expression. In parallel experiments, the same *in vivo* PR antagonist treatments also blocked NPY stimulation of GnRH release *in vitro*. Together our findings reveal that 1) Y1r mRNA levels are increased during the late morning and afternoon of proestrus; 2) Y1r mRNA levels are similarly increased by E_2 , and to an even greater extent by additional P; and 3) PR antagonism blocks both increased Y1r mRNA and induction of GnRH responsiveness to NPY. These observations support the idea that E_2 up-regulates GnRH neuronal responses to NPY through stimulation of Y1r gene expression, and that E_2 's actions are mediated by the induction and subsequent activation of PRs. (*Endocrinology* **141**: 3319–3327, 2000)

NEUROPEPTIDE Y (NPY) is critically important in the neural regulation of reproductive hormone secretions (1). The peptide is released at hypothalamic synapses to regulate the neurosecretion of GnRH (2–4) and into the hypophysial portal vasculature to modulate GnRH-induced gonadotropin secretion (5, 6). Neuropeptide Y's facilitating actions at both sites are important in mediating ovarian feedback signals for stimulating preovulatory GnRH and gonadotropin surges (5, 7, 8).

Within the hypothalamus, there are two mechanisms through which NPY may augment GnRH release during the initiation of midcycle surges. One is through enhancement of NPY production and secretion, as evidenced by findings that NPY gene expression (9, 10), NPY concentrations (11), and NPY release (12) are increased during the initiation of LH surges. A second mechanism involves a major up-regulation of tissue responsiveness to NPY, as stimulatory effects of NPY on GnRH release are dramatically increased just before GnRH surges (4). Cellular mechanisms mediating this up-regulatory process are not known, although they clearly involve some action of estrogen (E_2) on hypothalamic neurons (2, 13, 14).

These studies were designed to ascertain the cellular mechanisms through which E_2 up-regulates NPY's actions

on GnRH release. Specifically, we tested whether E_2 's up-regulatory effects are mediated by its ability to augment NPY receptor gene expression. Pharmacological studies implicate the NPY Y1 receptor (Y1r) subtype in stimulation of GnRH release by NPY (15, 16). The Y1r is a G protein-coupled receptor expressed in the arcuate and periventricular nuclei, preoptic area, and other hypothalamic sites known to be important in regulating GnRH neurosecretion (17). The highest concentrations of Y1r messenger RNA (mRNA) are found in the arcuate nucleus (18), where E_2 -responsive, progesterone (P) receptor (PR)-containing neurons are innervated by NPY neurons (19, 20). We therefore examined regulation of Y1r gene expression in tissues, including all of the foregoing hypothalamic areas, focusing on possible E_2 actions during the preovulatory period.

These studies also examined specific intracellular pathways through which E_2 may regulate NPY Y1r gene expression. E_2 treatments stimulate expression of PRs in brain (21), where they may subsequently be activated by P itself or by intracellular messengers derived from neurotransmitter signals initiated at the neuronal membrane (22, 23). In either case, *viz.* in liganded or unliganded states, activation of PRs previously induced by E_2 appears to be integral to neuroendocrine processes leading to gonadotropin surges, as shown by findings that E_2 treatments fail to induce LH surges in PR-knockout (PRKO) mice (24). We therefore considered the possibility that any actions of E_2 on Y1r expression and GnRH responsiveness to NPY may be mediated by activation of E_2 -induced PRs.

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Materials and Methods

Animals

Female Sprague Dawley rats were obtained from Charles River Laboratories, Inc. (Portage, WI) at 8–10 weeks of age and were maintained in temperature-controlled (23–25 C) and light-controlled (14-h light/10-h dark cycle, lights on at 0700 h) rooms. Animals were fed standard laboratory rat chow and had access to water *ad libitum*. The estrous cycles of the rats were monitored via examination of vaginal histology. Rats displaying at least two consecutive 4-day estrous cycles were used in the experiments.

Reagents

Rat NPY and BIBP3226 were purchased from Peninsula Laboratories, Inc. (Belmont, CA), and medium 199 (without phenol red), bacitracin, gentamicin, and synthetic oligonucleotides were obtained from Life Technologies, Inc. (Grand Island, NY). Benzyl benzoate, sesame oil, estradiol benzoate (E₂B), and P were purchased from Sigma (St. Louis, MO). The PR antagonists, RU486 and ZK98299, were gifts from Roussel-UCLAF (Romainville, France) and Dr. Klaus Stoeckmann (Schering AG, Berlin, Germany), respectively. *Taq* DNA polymerase was purchased from Fisher Scientific (Chicago, IL), and T4 DNA ligase, *Hind*III, T7 RNA polymerase, RQ1 deoxyribonuclease I (DNase I), deoxy (d)-NTPs, RNasin, and AMV-RT were supplied by Promega Corp. (Madison, WI). Other suppliers included BIO-101 (La Jolla, CA) for the GeneClean SPIN DNA isolation kit, Invitrogen (Carlsbad, CA) for the TA cloning kit (including pCRTMII vector), NEN Life Science Products (Boston, MA) for [α -³²P]dATP, and Amersham Pharmacia Biotech (Arlington Heights, IL) for Sequenase Kit 2.1.

Tissue superfusion

For superfusion experiments, tissues were obtained from female rats as previously described (4). At 1400 h on the afternoon of proestrus, rats were decapitated, and tissues containing median eminence and arcuate nucleus (ME-ARC) were quickly dissected away from the brain and placed into superfusion chambers (Brandel 6000 superfusion apparatus, Brandel, Gaithersburg, MD) containing 300 μ l superfusion medium. The superfusion medium consisted of medium 199 supplemented with 1.25 g/liter bacitracin, 0.003% BSA, and 20 mg/liter gentamicin and was equilibrated with 5% CO₂/95% O₂ before use. The medium was pumped through the warmed superfusion chamber (37 C) at a rate of 7 ml/h. In each experiment, the tissues were allowed to equilibrate in the system for 45 min, and thereafter continuous superfusate fractions were collected over 8-min intervals for a total of 3 h. NPY challenges were administered after a 45-min baseline collection period, and consisted of an 80-min infusion of 10⁻⁷ M NPY in superfusion medium. This concentration of NPY was chosen as an intermediate one based upon previous *in vitro* experiments that examined the effects of NPY on the ME (4, 25) and immortalized GnRH-producing neurons in culture (16). Control treatments consisted of infusions of medium alone. After NPY or control infusions, tissue viability was assessed by infusion of medium containing a depolarizing stimulus (60 mM KCl). All superfusate fractions were boiled for 30 min immediately after collection and stored at -20 C for subsequent GnRH RIA. Only data obtained from tissues still viable at the end of experiments were used for analysis (viable ME-ARC tissues responded to KCl with robust GnRH releases).

In initial experiments, the selective NPY Y1r antagonist BIBP3226 (8, 26) was used to assess the involvement of the Y1r subtype in the effects of NPY on GnRH release. For 15 min before NPY application and throughout the 80-min NPY challenge period, half of the superfusion chambers were superfused with medium containing 5 μ g/ml BIBP3226. The dose of BIBP3226 was chosen on the basis of the previous demonstration that it is a saturating concentration yet does not produce appreciable binding to Y2 receptors (27). Control chambers were superfused with either no NPY challenge or medium containing 5 μ g/ml BIBP3226 without NPY challenge.

Subsequent superfusion experiments tested the effects of *in vivo* treatment with PR antagonists, RU486 and ZK98299 (dissolved in 1:4 benzyl benzoate-sesame oil), on *in vitro* NPY-induced GnRH release. The anti-progestin ZK98299 exhibits less antigluccorticoid activity than does RU486 (28), so results were obtained using both compounds for com-

parison. Female rats were administered 6 mg/kg of either compound through sc injection or were given oil vehicle only at 0730 h on the morning of proestrus. Animals were killed at 1400 h, the ME-ARC tissues were collected for superfusion, and NPY challenges were supplied as described above.

RIA

Concentrations of GnRH in superfusion fractions were determined by RIA using EL-14 antiserum supplied by Dr. Martin J. Kelly, Oregon Health Sciences University (Portland, OR). The sensitivity of the assay was 0.2 pg/tube, and the intraassay coefficient of variation at the 1.0 pg level was 16%.

Measurement of hypothalamic Y1r mRNA levels by competitive RT-PCR

A competitive RT-PCR protocol (29, 30) was devised to quantify hypothalamic NPY Y1r mRNA levels. The competitive RT-PCR method we used was found to measure accurately as little as 25 attomoles (amol) Y1R mRNA in test samples.

RNA preparation. Animals were decapitated at scheduled time points, and hypothalamic tissue was quickly removed and placed on dry ice. Each excised tissue contained the mediobasal hypothalamus and preoptic area. Tissues were stored at -70 C until RNA extraction and RT-PCR procedures. For extraction of RNA, tissues were homogenized in Tri-Reagent using a Polytron homogenizer (Brinkmann Kinematica, Westbury, NY), and RNA was subsequently extracted by phenol/chloroform according to the Tri-Reagent manufacturer's protocol. All RNA samples were treated with RQ1 DNase I, and a RT-PCR test was performed to ensure that the RNA samples were of good integrity and were free of DNA contamination. RNA samples were stored at -70 C before use.

Preparation of competitor RNA (cRNA). The cRNA used as competitive standard was synthesized using Y1r DNA encoding the intron region and was 546 bp as opposed to the 436 bases of the corresponding region of Y1r mRNA (Fig. 1A). Rat DNA was extracted from rat tissue with Tri-Reagent and purified with phenol/chloroform. The possible existence of alternative forms of NPY Y1r mRNA transcripts was taken into consideration when designing primers for the PCR protocol. In the mouse, two isoforms of Y1r are generated by alternative splicing the 3-terminal of the transcripts: Y1 α and Y1 β . Y1 β is only expressed during embryonic stage and is not expressed in brain (31). In the human, multiple promoters regulate the tissue-specific expression of the Y1r gene. Alternative sequence transcribed from 1A, 1B, and 1C exons could appear in the final Y1r mRNA (32). In our experiment the primers for RT-PCR were designed according to the sequence spanning the exon 2 and exon 3 region of the Y1r gene, so that the RT-PCR product would reflect the overall mRNA levels of functional Y1r transcripts, including any possible corresponding alternative rat Y1R mRNA transcripts. According to known NPY Y1r gene sequences (33–36), primers were composed as follows: sense primer, ATC CTC ACA GGC TGT CTT AC (as depicted as Y1rE in Fig. 1A); and antisense primer, ATA GTC TCG TAG TCG TCG TC (shown as Y1rF in Fig. 1A). The primers Y1rE and Y1rF spanned the intron existing between exon 2 and exon 3 of the NPY Y1r gene. PCR amplification of the product was performed with 50 pmol of each primer and 50 μ g rat DNA (preheated at 95 C for 5 min, then 93 C for 1 min, 65 C for 2 min, 72 C for 2 min; 30 cycles). The PCR product was separated on a 1% agarose gel, and a DNA band of 546 bp was excised and purified with GeneClean Spin kit (BIO 101, Vista, CA). The purified DNA was ligated into pCRII vector (Invitrogen, Carlsbad, CA), and the sequence of the resultant clone was verified by DNA sequencing. Transformed cells were cultured, and the plasmid DNA was purified and sequenced. The desired clone was selected, in which a 546-bp DNA segment had been inserted into the vector downstream of the T7 promoter. The intron was confirmed to be 110 bp. The pCRII-Y1rE/F plasmid DNA was linearized with *Hind*III (Promega Corp.), separated on a 1% agarose gel, and purified. Using T7 RNA polymerase (Promega Corp.), cRNA was produced through *in vitro* transcription with linearized plasmid DNA. The cRNA sequence is identical to the corresponding part of NPY Y1r mRNA, with an additional 110-base internal segment

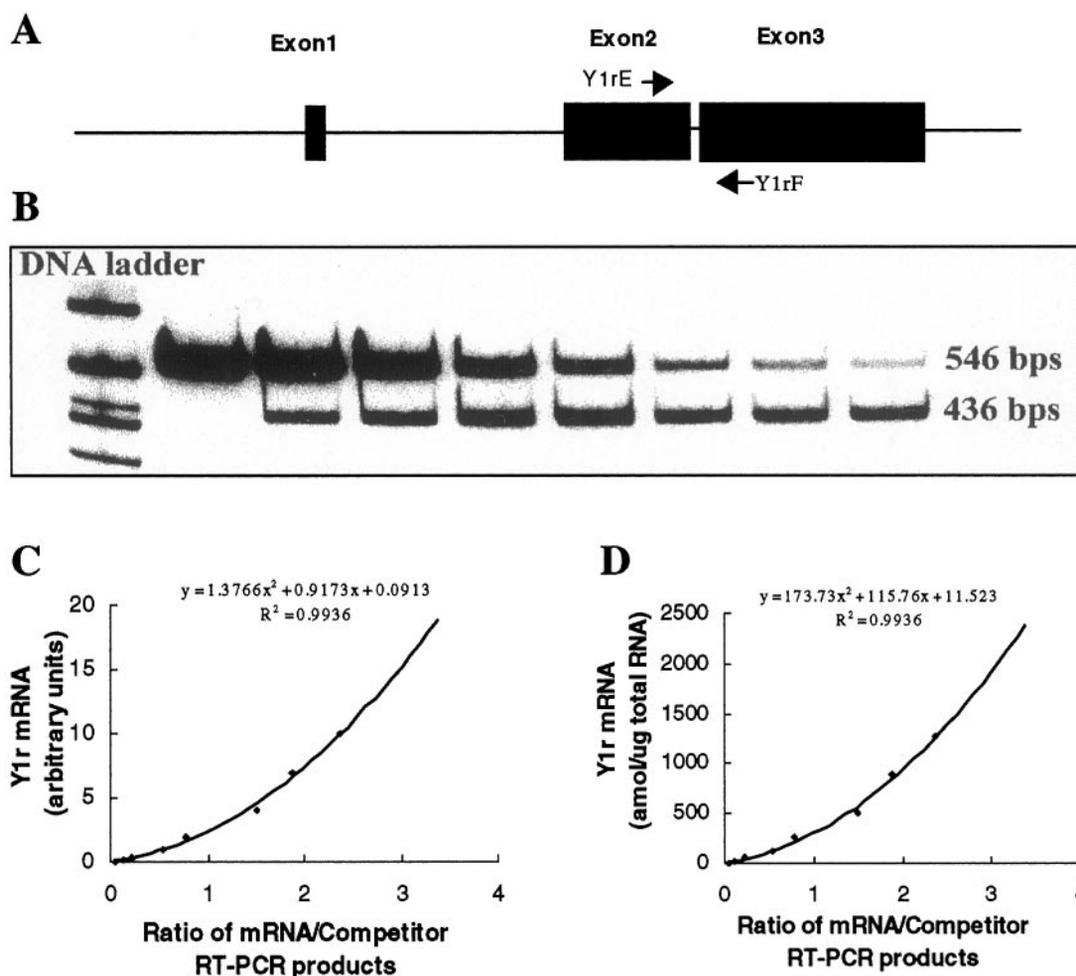


FIG. 1. Design and validation of competitive RT-PCR assay for quantitation of NPY Y1r mRNA. A, The gene structure of rat NPY Y1r. Y1rE and Y1rF denote sequences corresponding to the primers used in the construction of cRNA standard and competitive RT-PCR. B, Autoradiogram showing a RT-PCR-generated standard curve with cRNA standard (546 bp) and Y1r mRNA (436 bp). With 50 pg cRNA in each tube, 0, 0.2, 0.4, 1, 2, 4, 7, and 10 μ g of the same pooled RNA (containing a high concentration of Y1r mRNA) were added, respectively, to each tube, from left to right. Radioactively end-labeled pGEM4 digested with *Dde*I was used as DNA reference ladder. C, Standard curve. The Y1r mRNA/cRNA RT-PCR product ratio is plotted against Y1r mRNA levels (arbitrary units). D, Standard curve. The Y1r mRNA/cRNA RT-PCR product ratio is plotted against Y1r mRNA, expressed as attomoles (10^{-18} mol).

transcribed from the intron sequence between Y1r gene exons II and III. The cRNA was digested twice with RQ1 DNase I to eliminate any residual DNA contamination. RT-PCR was performed using primers Y1rE and Y1rF to check the integrity and purity of the cRNA. In RT-PCR performed in the presence of both Y1r mRNA and cRNA, the RT-PCR product of the cRNA could be visualized as a band of 546 bp and easily distinguished from that of NPY Y1r mRNA (436 bp). cRNA was kept at -70 C until use.

Standard curve for competitive RT-PCR. A RNA pool was prepared from rat hypothalamus and frontal cortex, where Y1r mRNA is enriched. The RNA was digested with RQ1 DNase I to eliminate DNA contamination. RT was performed in 20- μ l reactions, with 5 U AMV-RT, 0.4 μ l 100 mM dithiothreitol, 20 U RNasin, 1 μ l 500 μ g/ml random hexamer primer, 2 μ l 10 mM dNTPs, 2 μ l 10 \times assay buffer B (Fisher Scientific, Fairlawn, NJ), and 3 μ l 25 mM MgCl₂ plus RNA and cRNA. In a series of tubes containing 50 pg cRNA, 0, 0.2, 0.4, 1.0, 2.0, 4.0, 7.0, or 10.0 μ g DNA-free pooled RNA (containing a high concentration of Y1r mRNA) was added to each tube, respectively. Tubes were incubated at 42 C for 75 min, followed by incubation at 95 C for 5 min. The PCR reaction was performed in a 50- μ l volume containing 3 μ l of each 20- μ l RT product, 7 μ l RT supplement mix (each 20 μ l containing 2 μ l 10 \times assay buffer B, 2 μ l 10 mM dNTPs, 3 μ l 25 MgCl₂, and 13 μ l dH₂O), 25 pmol primer Y1rE and Y1rF each, 4 μ l 10 \times assay buffer B, 3 μ l 25 mM MgCl₂, 0.75 U *Taq*

DNA polymerase, 0.1 μ l [³²P]dATP (NEN Life Science Products; 3000 Ci/mmol), and ddH₂O. The PCR conditions consisted of 25 cycles of denaturing at 93 C for 1 min, annealing at 65 C for 2 min, and extension at 72 C for 2 min, followed by a final extension period of 10 min at 72 C. The PCR products were separated by electrophoresis on 5% polyacrylamide (Fig. 1B). The intensity of radioactivity from the two DNA bands (436 and 546 bp) were analyzed (ImageQuant System, Molecular Dynamics, Inc., Sunnyvale, CA), and regression analysis was performed using Excel (Microsoft Corp., Redmond, WA). A standard curve for the competitive RT-PCR was constructed in which Y1r mRNA levels were expressed as arbitrary units and plotted against the mRNA/cRNA RT-PCR product ratios (Fig. 1C). When the input amounts of cRNA and Y1r mRNA are equimolar, the ratio of Y1r mRNA/cRNA RT-PCR products is predicted to be 0.7909. With a known amount of cRNA (50 pg, or 2.19×10^{-16} mol) in each reaction tube, the amount of corresponding Y1r mRNA level could be obtained through calculation. The corresponding Y1r mRNA levels were transformed from arbitrary units to mass, expressed in attomoles. In the final form of the standard curve, the ratio of mRNA/cRNA RT-PCR product was plotted against the total amount of Y1r mRNA (attomoles) in each reaction (Fig. 1D).

Measurement of NPY Y1r mRNA in tissues. RT was performed on samples using 1 μ g sample RNA in the presence of 50 pg cRNA, and PCR was performed and analyzed under the same conditions as those described

for the standard curve preparation. The NPY Y1r mRNA levels were calculated via the standard curve regression equation and expressed as attomoles of Y1r mRNA per μg total sample RNA. Y1 receptor mRNA data obtained from different assays were normalized by the assay value obtained from a common DNA-free RNA pool to permit direct comparison.

Competitive RT-PCR assay characteristics. Coefficients of variation for the assays at 150, 300, and 1500 amol were 6.99%, 5.43%, and 8.24%, respectively. The lower limit of sensitivity, as defined by 2 times the SD of 10.24695, was 20 amol. Values for NPY Y1r hypothalamic samples fell within the 200-2500 amol range of the assay.

Animal protocols. To examine hypothalamic Y1r mRNA during the estrous cycle, 8–10 rats were killed at 1000, 1400, and 1800 h on metestrus, diestrus, proestrus, and estrus; an additional group of rats was killed at 1200 h on proestrus. To assess the effects of ovarian steroids on hypothalamic Y1r mRNA levels, groups of 8–10 rats were ovariectomized (OVX) and 6 days later were treated with sc injection of 30 μg E_2B or oil vehicle at 0900 h. Another 48 h later (day 8), animals received either no additional treatment and were killed at 0900 h or received sc injection of oil vehicle or 5 mg P at 0900 h and were killed at 1800 h the same day. The role of PRs in the regulation of Y1r gene expression was assessed using PR antagonists in proestrous rats. Each of these rats received no treatment, oil vehicle, RU486 (6 mg/kg), or ZK98299 (6 mg/kg) sc injection at 0730 h on proestrous morning and was killed at 1200 h on the same day. In all experiments, the hypothalamic tissues from all animals were stored at -70°C for subsequent RNA isolation and competitive RT-PCR assay as described.

Statistical analysis

In the ME-ARC superfusion experiments, the data from groups of different treatments (NPY, BIBP3226, and PR antagonists ZK98299/RU486) were compared with corresponding control values. The differences in levels of GnRH release among groups were analyzed by two-way ANOVA with repeated measures (match values by superfusion sample collection times), and *post-hoc* analysis were performed using Bonferroni's test. Statistical analysis of differences in Y1r mRNA levels among different groups were assessed by one-way ANOVA followed by Tukey's *post-hoc* test. Differences were considered significant at $P < 0.05$.

Results

Y1 receptors mediate NPY-induced GnRH release

Initial *in vitro* pharmacological experiments were conducted to confirm the involvement of the NPY Y1r subtype (15, 16, 37) in mediating NPY effects on GnRH release. Introduction of NPY (10^{-7} M) into the superfusion medium evoked a significant increase in GnRH release from ME-ARC fragments (Fig. 2A). As shown previously (4, 25), the NPY challenge induced a release of GnRH that commenced about 30 min after initiation of the stimulus and gradually reached a level approximately 2-fold greater than the corresponding control values. The facilitating effects of NPY were completely prevented by preexposure and coexposure of tissues to the specific NPY Y1r antagonist, BIBP3226 at a concentration of 5 $\mu\text{g}/\text{ml}$ (Fig. 2B). The Y1r antagonist was without effect on *in vitro* GnRH release in the absence of NPY (Fig. 2C). To confirm the viability of each tissue in superfusion experiments, a depolarizing stimulus consisting of medium containing 60 mM K^+ was delivered to the chambers after termination of the NPY challenge. For all experimental conditions, the superfused tissues responded to the K^+ stimulus with a significant increase in GnRH release ($P < 0.001$) for all groups (Fig. 2, A–C). Moreover, the effects of the K^+ stimulus were similar in all tissues, indicating the lack of any overt

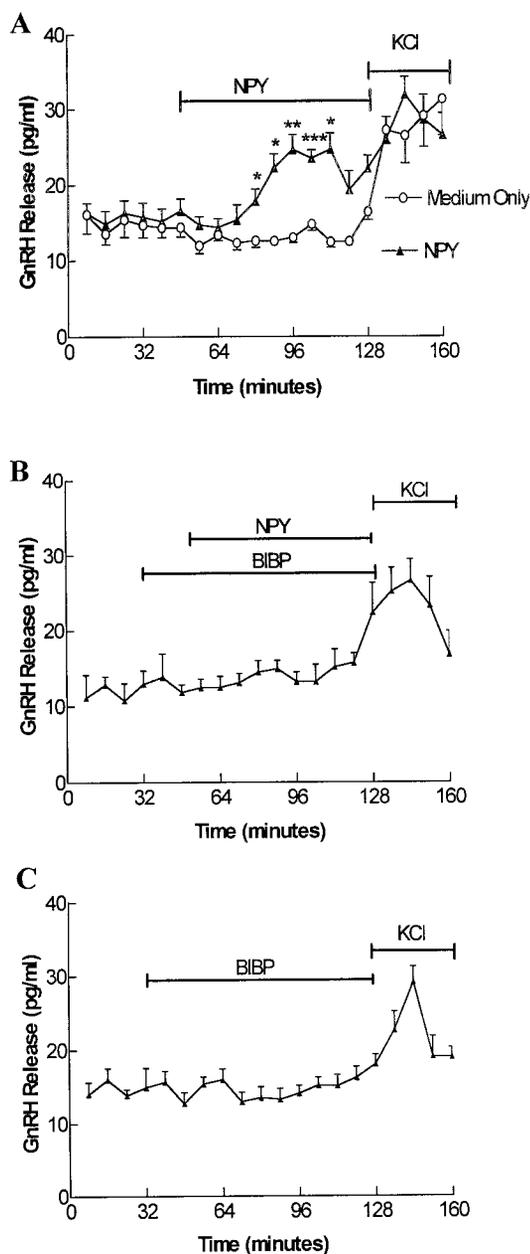


FIG. 2. Effects of NPY on GnRH release from ME-ARC tissues during *in vitro* superfusion. Horizontal bars above the graphs depict corresponding intervals during that period the indicated test substances were applied in superfusion medium. Continuous superfusate fractions were collected at 8-min intervals and analyzed for GnRH content by RIA. A, Infusion of 10^{-7} M NPY induced a significant increase in GnRH release from ME-ARC at 32–64 min after the onset of NPY challenge (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; $n = 13$ for experimental and control groups). B, Stimulation of GnRH release is completely blocked by pre- and cotreatment with 5 $\mu\text{g}/\text{ml}$ NPY Y1r antagonist BIBP3226 ($n = 8$). C, BIBP3226 alone is without effect on GnRH release ($n = 9$).

toxicity of any of the treatment regimens on GnRH neurons in experiments.

NPY Y1r gene expression during estrus cycle

The competitive RT-PCR method was used to quantify hypothalamic NPY Y1r gene expression. Throughout the

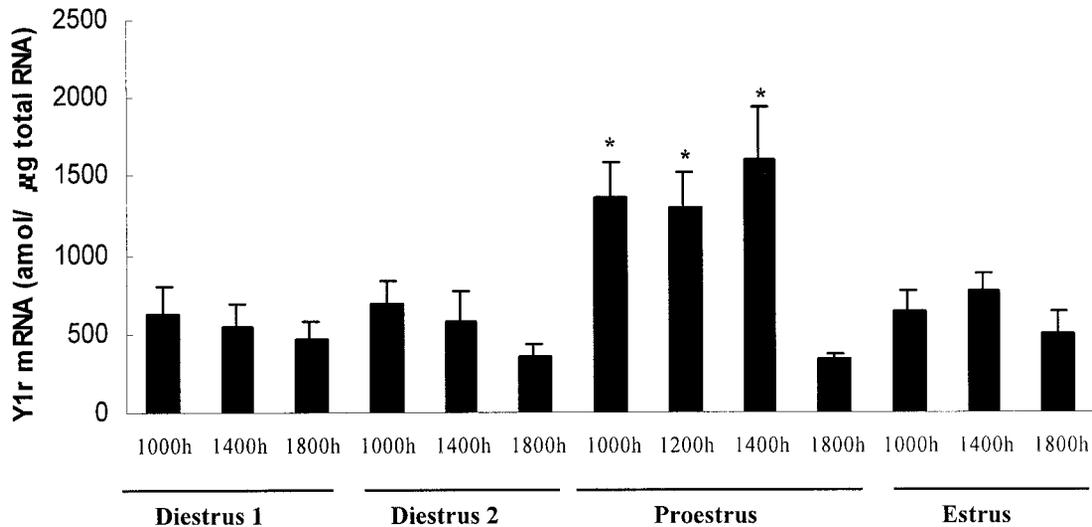


FIG. 3. Hypothalamic NPY Y1r mRNA levels during the estrous cycle. Data are represented as the mean ± SEM (n = 8–10). Compared with all other stages of the estrous cycle, hypothalamic NPY Y1r mRNA levels were significantly higher at 1000, 1200, and 1400 h of proestrus (*, P < 0.05).

course of the estrous cycle, the Y1r mRNA levels (Fig. 3) remained low and unchanged in tissues obtained at all three sample times on estrus, metestrus, and diestrus (350–650 amol/µg total RNA). At 1000, 1200, and 1400 h on proestrus, however, the hypothalamic Y1r mRNA levels were significantly greater than those observed in tissues obtained at all other time points (Fig. 3). By 1800 h proestrus, the mean Y1r mRNA level was again found to be diminished, reaching the lowest value of any time point throughout the estrous cycle. Overall, Y1r gene expression was elevated on the morning (1000 h) and early afternoon (1200 and 1400 h) of proestrus, but not before or after this period. In several previous studies we documented that this period coincides with the culmination of preovulatory estrogen secretions and immediately precedes the onset of gonadotropin surges (38).

Effects of ovarian steroids on NPY Y1r gene expression

To directly test the hypothesis that ovarian steroids can stimulate Y1r gene expression, OVX rats were treated with oil vehicle, E₂B, P, or E₂B followed by P, and hypothalamic tissues were obtained at morning (0900 h) and afternoon (1800 h) points and analyzed for Y1r mRNA content. Y1r mRNA levels were low at both 0900 and 1800 h in OVX rats (Fig. 4), and the absolute values closely approximated those observed in estrous, metestrous, and diestrous rats in the previous experiment. In the OVX rats receiving E₂B, however, Y1r mRNA levels were significantly elevated (P < 0.05) compared with corresponding values in the OVX oil-treated rats. Additional treatment of OVX E₂B-primed rats with P produced an even greater increase in Y1r mRNA at 1800 h (P < 0.01) compared with that in OVX oil-treated animals (Fig. 4). In animals that received P without prior E₂B priming, Y1r mRNA levels were no different from those in OVX oil-treated rats.

PR antagonism blocks NPY Y1r gene expression

The results obtained in OVX, steroid-primed rats suggested that PR activation can provoke a major enhancement

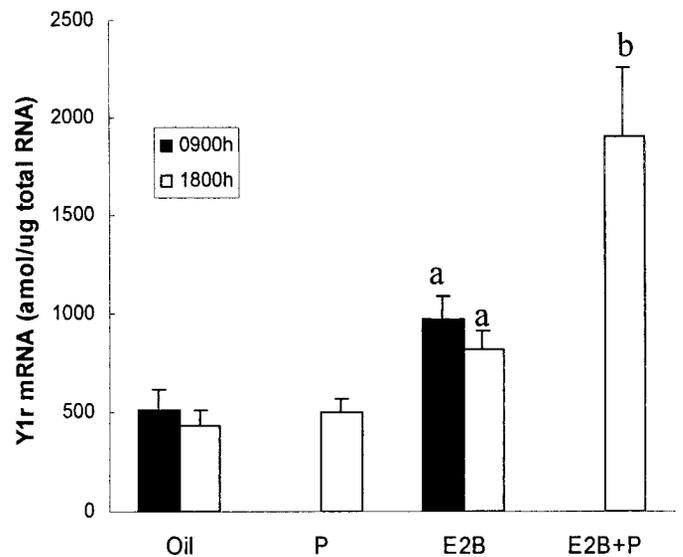


FIG. 4. Effects of *in vivo* E₂B and P treatments on hypothalamic NPY Y1r mRNA levels. See *Materials and Methods* for treatment details. E₂B treatment significantly increased hypothalamic NPY Y1r mRNA levels (superscript a denotes significantly different compared with results from OVX, oil- or P-treated rats, P < 0.05; n = 8–10/group). P treatment alone had no significant effect on hypothalamic NPY Y1r mRNA levels. Combined treatment of E₂B and P increased hypothalamic NPY Y1r mRNA to the greatest extent (b; P < 0.01; n = 8–10/group, compared with results from OVX, oil- or P-treated rats).

of Y1r gene expression. We therefore tested the possibility that PR activation in intact proestrous rats mediates the increase in Y1r mRNA levels that occurs on that day of the cycle. Proestrous rats were untreated, treated with oil vehicle, or treated with one of two PR antagonists, ZK98299 or RU486, at 0730 h and subsequently killed at 1200 h, a time at which Y1r mRNA levels were previously shown to be elevated in untreated proestrous rats. Data from the assay of Y1r mRNAs in these groups of tissues are depicted in Fig. 5. Pretreatment with either RU486 or ZK98299 led to significant

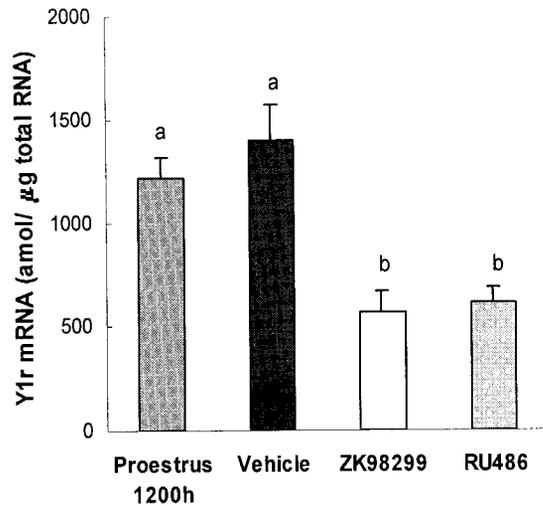


FIG. 5. Effects of *in vivo* PR antagonism on hypothalamic NPY Y1r mRNA levels. The application of PR antagonist, RU486 or ZK98299 (6 mg/kg), at 0730 h on proestrus effectively blocked the increase in hypothalamic Y1r mRNA at 1200 h (different lowercase letters show a significant difference, $P < 0.05$; $n = 8-10$ /group). The Y1r mRNA levels observed in the untreated or vehicle-treated proestrus rats were comparable to those reported in previous experiments (Fig. 3).

reductions in the Y1r mRNA levels compared with those observed in the untreated or oil vehicle-treated groups ($P < 0.01$; Fig. 5). Values in the untreated or oil vehicle-treated groups closely resembled the values observed in 1200 h proestrous animals in the previous experiment; similarly, Y1r mRNA levels after PR antagonist treatment were indistinguishable from the low values obtained at cycle times other than proestrous morning or early afternoon.

PR antagonism blocks NPY-induced GnRH release

Additional superfusion experiments were conducted to assess the degree to which PR activation may also mediate increased GnRH responsiveness to NPY. We reasoned that if enhanced Y1r gene expression on proestrus is dependent upon PR activation, and increased GnRH responsiveness to NPY is, in turn, dependent upon enhanced Y1r gene expression, then PR antagonism should block or attenuate the ability of NPY to stimulate GnRH release. Proestrous rats were treated with oil vehicle, RU486, or ZK98299 in the same way as in the previous experiment, and the ME-ARC tissues were removed for superfusion experiments. The standard NPY stimulus (10^{-7} M NPY in medium for a duration of 80 min) was delivered to the tissues, and the patterns of GnRH release in response to the NPY challenges were assessed. Pretreatment with either PR antagonist resulted in a complete loss of responsiveness to the NPY stimulus (Fig. 6). Although NPY again increased the GnRH release rate in tissues from the oil-vehicle treated animals, the same NPY stimulus produced no effect in tissues of rats pretreated with either PR antagonist. The loss of responsiveness to NPY in tissues of PR antagonist-treated rats was not accompanied by any diminution in the magnitude of the tissue's responsiveness to depolarization with medium containing 60 mM KCl.

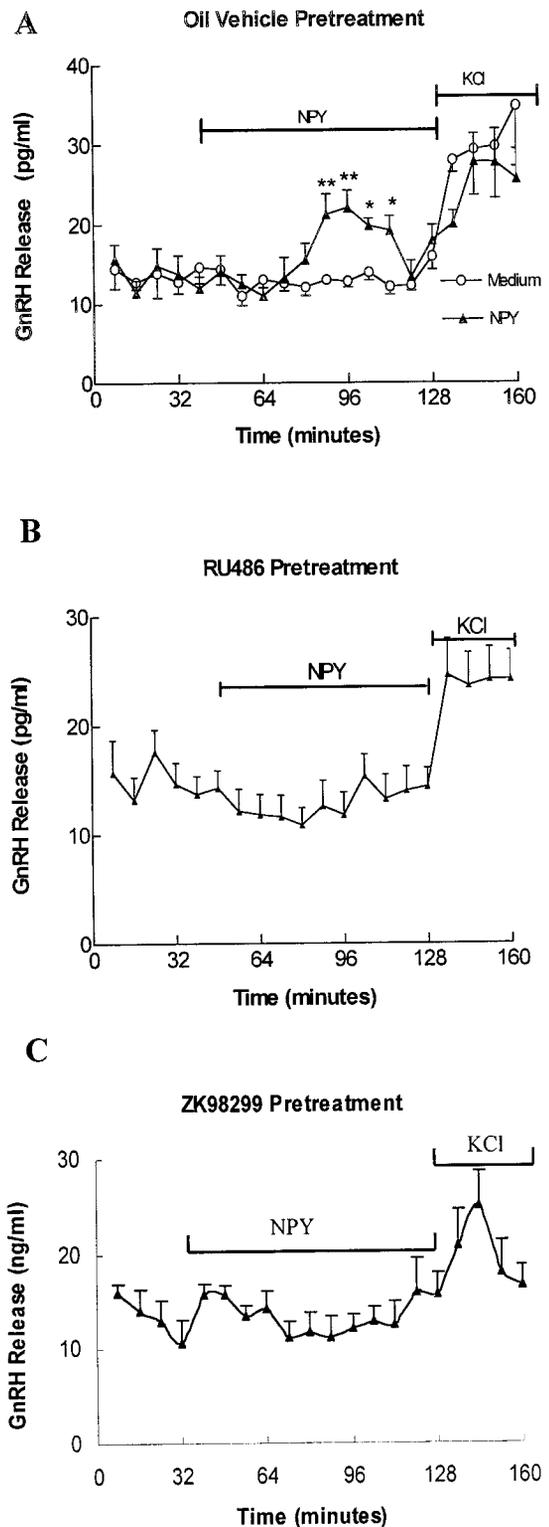


FIG. 6. Effects of PR antagonism on *in vitro* GnRH release responses to NPY stimulation. A, In vehicle-treated rats, NPY was again found to elevate GnRH release ($n = 8-10$ for NPY and control groups; *, $P < 0.05$; **, $P < 0.01$). B and C, Pretreatment of rats with either RU486 or ZK98299 at 0730 h on proestrus completely blocked NPY's ability to induce GnRH release in ME-ARC superfusion.

Discussion

In female rats, the midcycle surge of gonadotropins is triggered by the release of a GnRH surge into the hypothalamic hypophysial portal vasculature. There are two major determinants of the GnRH surge: exposure of the hypothalamus to preovulatory E_2 secretions, and transmission of neural signals for the surge from the 24-h neural clock. It is believed that the major function of E_2 in this regard is to couple the clock-derived signals to the neuronal circuitries governing GnRH release. It remains unclear, however, how E_2 may act in individual neurons to confer patency through appropriate signaling pathways. We assessed the hypothesis that one major action of E_2 is to up-regulate the expression of receptors for a neurotransmitter, *i.e.* NPY, which is known to mediate signals for initiation of GnRH surges. Our observations reveal that Y1r mRNA expression in hypothalamus is up-regulated on proestrus and stimulated by exogenous E_2 treatment. Moreover, these changes in Y1r mRNA expression occur only under conditions in which GnRH responsiveness to NPY is enhanced, *viz.* on proestrus (4) or in OVX rats after E_2 treatment (13). Taken together, these studies are consistent with the idea that E_2 induces Y1r expression and thereby confers greater GnRH responsiveness to NPY. We further propose that these actions of E_2 represent a major component of the neuroendocrine mechanisms governing release of preovulatory GnRH surges.

Central application of NPY antiserum was previously shown to attenuate LH surges in female rats (7), firmly establishing the requirement for hypothalamic NPY release in the generation of GnRH surges. The nature of this requirement appears to be 2-fold; in addition to the postsynaptic up-regulation of responsiveness to NPY suggested by our previous (4) and current work, presynaptic up-regulation of NPY gene expression (9) and release (5, 12) also occurs during the preovulatory period. Enhancement of NPY expression and NPY actions may occur in parallel, both being stimulated independently by E_2 . It is also possible that E_2 primarily stimulates the expression of NPY, and the resultant increase in NPY release leads to autologous up-regulation of postsynaptic responsiveness. We do not favor the latter possibility, however, because peak NPY release before gonadotropin surges (12) does not precede the period of maximal responsiveness to NPY (4). In any case, it appears that E_2 prompts complimentary changes at both pre- and postsynaptic sites, which together produce a robust facilitatory signal that ultimately reaches GnRH neurons.

It remains to be determined which neuronal phenotypes express E_2 -induced Y1rs, and which of these cells participate in modulation of GnRH surges. Immortalized GnRH neurons respond to NPY analogs in a manner pharmacologically consistent with Y1rs (16). It is therefore possible that NPY axon terminals release the peptide at synaptic junctions with GnRH neurons and facilitate GnRH release through receptors expressed on GnRH neurons. We have proposed that the Y1r mediates the effects of NPY, and that estrogen modulates the expression of the Y1rs. Thus, if NPY activates Y1rs in GnRH neurons, and the expression of Y1rs is regulated via ER activation, then GnRH neurons would be expected to express the ERs that would function in this manner. How-

ever, numerous reports have failed to detect appreciable ER expression in GnRH neurons (39–41), suggesting that the locus of action of E_2 is not within the GnRH neuronal population itself. This issue remains to be resolved, however, in light of a recent study (42) that used single cell PCR methods to detect ER mRNA in GnRH neurons.

Alternatively, ER activation and subsequent regulation of Y1r expression may occur within afferent circuitries that govern the activity of GnRH neurons. Recent studies (43) demonstrated little or no Y1-positive staining in GnRH cell bodies in the organum vasculosum of the lamina terminalis and preoptic area, although abundant Y1r-positive fiber and cell staining were observed throughout the region. Moreover, Y1r-positive fibers were located in close apposition to GnRH cell bodies. The Y1r (43, 44), estrogen receptor (39, 41, 45), and PR (19, 46–48) are also expressed in coextensive regions within the arcuate nucleus and adjacent regions within the mediobasal hypothalamus. It is therefore possible that NPY actions via E_2 -induced Y1rs are exerted in either preoptic or mediobasal hypothalamic cells functioning as afferents to GnRH neurons. To fulfill the requirements for being such interneurons, cells would receive projections from NPY neurons, express steroid receptors, and make synaptic contacts with GnRH neurons. Although no specific cell group has been unequivocally shown to possess all of these characteristics, candidate neuronal populations could include catecholaminergic (19, 20, 49, 50) and β -endorphinergic neurons (51–59).

We have demonstrated that E_2 treatments that up-regulate GnRH responses to NPY (13) also produce increases in Y1r gene expression. It is not clear how E_2 may stimulate Y1r expression, but one possibility is that E_2 may directly regulate transcription of the Y1r gene. Sequence analysis of the 5'-flanking region of the rat Y1r gene (32) has revealed the presence of consensus activating protein 1 and P/glucocorticoid response elements (PRE/GRE) sites in the promoter region of the gene, but no consensus estrogen response element site was identified. Detailed functional analysis of these putative regulatory elements, however, has not been carried out. Nevertheless, presence of consensus PRE/GRE sites suggests an alternative possibility, *viz.* that E_2 effects may be mediated by its ability to induce PRs (21), and that estrogen receptor-induced PRs may thereafter be activated and prompt increased transcription of the Y1r gene.

Our data are consistent with the hypothesis that E_2 's actions are mediated by PR induction, as PR antagonism blocked both the effects of NPY on GnRH release as well as the induction of Y1r gene expression in proestrous rats. Moreover, additional treatment of OVX E_2 -primed rats with P resulted in additional stimulation of Y1r gene expression compared with OVX rats treated with E_2 only. Indeed, stimulation of Y1r by combined treatment with E_2 and P exceeded the level of stimulation seen in tissues of proestrous rats. This may reflect the fact that surge-inducing regimens of E_2 and P necessarily produce prematurely elevated P levels (60) compared with intact proestrus rats (61). Thus, Y1r levels may be most augmented in the E_2 - and P-treated rats due to the peculiar characteristics of the early morning exogenous P signal required to amplify LH surges in OVX E_2 -primed rats. It is clear, nevertheless, that activation of PRs leads to

a robust amplification of Y1r mRNA expression, and blockade of PRs completely prevents the rise in Y1r mRNA levels that occurs in proestrus rats. The lack of effect of P alone, without E₂ priming, is probably due to the absence of E₂-induced PRs.

If E₂-induced PRs mediate stimulation of Y1r gene expression, then which intracellular signals activate the PRs toward this end? Circulating P, whether derived from the ovaries or adrenals, may function as the cognate ligand that initiates this process. There have been no reports, however, that circulating P levels exhibit signal changes in plasma levels throughout the morning of proestrus. Indeed, the increased expression of Y1r occurs by 1000 h on proestrous morning, whereas a detectable increase in P secretion does not occur until the onset of LH surge in the late afternoon (62, 63). Locally produced P in brain (64) might also activate E₂-induced PRs, although the low amounts of the steroid that are produced by the brain have been implicated most often in the manifestation of nongenomic P actions, independent of PR_A or PR_B activation. An alternative possibility is that E₂-induced PRs are *trans*-activated in an unliganded state by intracellular signals that arise from neurotransmitter receptor activation. There is now considerable evidence, for example, that dopamine receptor activation and subsequent cAMP formation can *trans*-activate unliganded PRs and thereby facilitate sexual behavior (23, 65). Similarly, GnRH self-priming appears to be mediated by ligand-independent activation of PRs in gonadotropes (22). It is thus possible that *trans*-activation of E₂-induced PRs in the unliganded state may occur as a result of neurally derived signals. Regardless of the mechanism, it is clear that induction of Y1r gene expression on proestrus as well as the induction of GnRH responsiveness to NPY are dependent upon PR *trans*-activation. It remains to be determined whether the *trans*-activated PR directly regulates Y1r gene transcription, perhaps at the putative PRE/GRE sites in the Y1r gene promoter, or whether the regulation occurs at the level of Y1r mRNA stability, Y1r mRNA translation, or via regulation of one or more intermediate genes.

The cellular mechanisms by which E₂ evokes GnRH surges have been difficult to study, given the likelihood that most E₂ actions on GnRH release are exerted indirectly, on interneurons rather than on GnRH neurons themselves. A variety of cellular actions of E₂ in hypothalamic neurons have been demonstrated, including the regulation of neurotransmitter gene expression, neurotransmitter receptor gene expression, receptor-G protein coupling, expression of neurotransmitter-synthesizing enzymes, ionic conductances, and expression of intracellular signaling molecules. Our findings suggest that E₂ can also exert an important cellular action that serves a permissive function in target brain cells; by stimulating PR expression, it endows E₂-responsive neurons with the capacity to respond to subsequent neural and endocrine signals. Ultimately, the E₂-induced PRs are *trans*-activated in a ligand-dependent or a ligand-independent manner and stimulate expression of the NPY Y1r receptor gene. In this manner, E₂ actions appear to permit heightened patency of signaling pathways leading to initiation of GnRH surges. Although we have shown that this neuroendocrine signaling mechanism may operate in the case of NPY and the Y1r

receptor, it remains to be determined whether other neurotransmitter receptor genes are regulated by E₂-induced PRs in an analogous manner, and whether these effects may similarly contribute to physiological events such as GnRH release on proestrus.

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