Re a t f H t a a f c Ne r e t de Y Y1 Rece t r Ge e E re d r t e E t r C c e: R e f P r e e r e Rece t r*

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

ABSTRACT

in vivo *in vitro*

(*Endocrinology* **141:**

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 nt i(si906 -1.1

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 (E_2B), 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, *Hin*dIII, 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-^{32}P]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 μl 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% CO_2/95% O_2 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*

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-μl reactions, with 5 U AMV-RT, 0.4 μl 100 mm dithiothreitol, 20 U RNasin, 1 μl 500 μg/ml random hexamer primer, 2 μl 10 mm dNTPs, 2 μl 10 $\times$ assay buffer B (Fisher Scientific, Fairlawn, NJ), and 3 μl 25 mm $MgCl_2$ 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 μg 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-μl volume containing 3 μl of each 20-μl RT product, 7 μl RT supplement mix (each 20 μl containing 2 μl 10 $\times$ assay buffer B, 2 μl 10 mm dNTPs, 3 μl 25 $MgCl_2$, and 13 μl dH_2O), 25 pmol primer Y1rE and Y1rF each, 4 μl 10 $\times$ assay buffer B, 3 μl 25 mm $MgCl_2$, 0.75 U *Taq* DNA polymerase, 0.1 μl [^{32}P]dATP (NEN Life Science Products; 3000 Ci/mmol), and ddH_2O. 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

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.

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 estrus 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, E_2B, P, or E_2B 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 E_2B, however, Y1r mRNA levels were significantly elevated ($P <$

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 minuction2PuctioneT; TD[(ity7 03n)Tj/T1_0 1 Tfo-241(wa/T1_0res-)]TJT*[_0re
340241(RU43402ei)-333(m40228(al402(were)-291(il402[(Nulcle-tr4026(or)402(or)402]TJle)-213(GnRH)-2196(to)-.os,)-19

D c

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 possibil-2

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 E_2 priming, is probably due to the absence of E_2-induced PRs.

If E_2-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 E_2-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 E_2-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 E_2-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 E_2 evokes GnRH surges have been difficult to study, given the likelihood that most E_2 actions on GnRH release are exerted indirectly, on interneurons rather than on GnRH neurons themselves. A variety of cellular actions of E_2 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 E_2 can also exert an important cellular action that serves a permissive function in target brain cells; by stimulating PR expression, it endows E_2-responsive neurons with the capacity to respond to subsequent neural and endocrine signals. Ultimately, the E_2-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, E_2 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 E_2-induced PRs in an analogous manner, and whether these effects may similarly contribute to physiological events such as GnRH release on proestrus.

Ac ed e t

The authors thank Brigitte Mann for her expert technical assistance with hormone measurements.

Refe e ce

1. **Levine J** 1997 New concepts of the neuroendoocrine regulation of gonadotropin surges in rats. Biol Reprod 56:293–302
2. **Crowley WR, Kalra SP** 1987 Neuropeptide Y stimulates the release of luteinizing hormone-releasing hormone from medial basal hypothalamus in vitro: modulation by ovarian hormones. Neuroendocrinology 46:97–103
3. **Woller MJ, Terasawa E** 1991 Infusion of neuropeptide Y into the stalk-median eminence stimulates *in vivo* release of luteinizing hormone-release hormone in gonadectomized rhesus monkeys. Endocrinology 128:1144–1150
4. **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
5. **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
6. **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
7. **Wehrenberg WB, Corder R, Gaillard RC** 1989 A physiological role for neuropeptide Y in regulating the estrogen/progesterone induced luteinizing hormone surge in ovariectomized rats. Neuroendocrinology 49:680–682
8. **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
9. **Bauer-Dantoin AC, Urban JH, Levine JE** 1992 Neuropeptide Y gene expression in the arcuate nucleus is increased during preovulatory luteinizing hormone surges. Endocrinology 131:2953–2958
10. **Sahu A, Crowley WR, Kalra SP**