Behavioral/Systems/Cognitive

Estrogen-Induced μ-Opioid Receptor Internalization in the Medial Preoptic Nucleus Is Mediated via Neuropeptide Y-Y_1 Receptor Activation in the Arcuate Nucleus of Female Rats

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The endogenous peptides β-endorphin (β-END) and neuropeptide Y (NPY) have been implicated in regulating sexual receptivity. Both β-END and NPY systems are activated by estrogen and inhibit female sexual receptivity. The initial estrogen-induced sexual nonreceptivity is correlated with the activation and internalization of μ-opioid receptors (MORs), in the medial preoptic nucleus (MPN). Progesterone reverses the estrogen-induced activation/internalization of MOR and induces the sexual receptive behavior lordosis. To determine whether NPY and endogenous opioids interact, we tested the hypothesis that estrogen-induced MOR activation is mediated through NPY-Y_1 receptor (Y_1R) activation. Retrograde tract tracing demonstrated Y_1R on β-END neurons that projected to the MPN. Sex steroid modulation of MOR in the MPN acts through NPY and the Y_1R. Estradiol administration or intracerebroventricular injection of NPY activated/internalized Y_1R in the arcuate nucleus and MOR in the MPN of ovariectomized (OVX) rats. Moreover, the selective Y_1R agonist [Leu31, Pro34]-Neuropeptide Y (LPNY) internalized MOR in the MPN of OVX rats. The Y_1R antagonist (Cys^{31}, Nva^{34})-Neuropeptide Y (27–36)$_2$ prevented estrogen-induced Y_1R and MOR activation/internalization. NPY reversed the progesterone blockade of estradiol-induced Y_1R and MOR internalization in the arcuate nucleus and MPN, respectively. Behaviorally, LPNY inhibited estrogen plus progesterone-induced lordosis, and the MOR-selective antagonist D-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr amide reversed LPNY-induced inhibition of lordosis. These results suggest that a sequential sex steroid activation of NPY and MOR circuits regulates sexual receptivity.

Key words: **internalization; reproduction; hypothalamus; receptors; lordosis; estrogen; progesterone**

Introduction

Two neuropeptides, neuropeptide Y (NPY) and β-endorphin (β-END), have been implicated in the transduction of steroidal information into the CNS and modulate reproductive function. Estrogen receptors have been colocalized in β-END and NPY immunoreactive neurons (Morrell et al., 1985; Sar et al., 1990), and estradiol regulates the expression of both NPY and β-END in the arcuate nucleus of the hypothalamus (ARH) (Petraglia et al., 1982; Wardlaw et al., 1982; Wilcox and Roberts, 1985; Baskin et al., 1995; Priest and Roberts, 2000).

β-END-containing neurons are concentrated in the ARH (Finley et al., 1981; Micevych and Elde, 1982; Khachaturian et al., 1983), which project throughout the neuroaxis including the medial preoptic nucleus (MPN) (Cheung and Hammer, 1995). Central injections of either NPY or β-END inhibit reproductive behavior in rats and hamsters when primed with estradiol and progesterone (Sirinathsinghji et al., 1983; Wiesner and Moss, 1984; Clark et al., 1985; Gorzalka et al., 1997; Torii et al., 1999; Corp et al., 2001). Furthermore, infusion of β-END into the MPN inhibited lordosis (Sirinathsinghji, 1986), suggesting that β-END is an endogenous ligand of the μ-opioid receptor (MOR) in this nucleus.

The MOR, a G-protein-coupled receptor, undergoes agonist-induced receptor internalization (von Zastrow et al., 1993). Internalization can be used as an assay for activation of hypothalamic MOR (Eckersell et al., 1998; Sinchak and Micevych, 2001, 2003) and has been correlated with an inhibition of lordosis after estradiol treatment, microinjections, exogenous [D-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin or endogenous MOR agonists, and endomorphin-1 (Eckersell et al., 1998; Sinchak and Micevych, 2001). Progesterone reversal of estradiol-induced MOR activation/internalization is correlated with a facilitation of lordosis (Sinchak and Micevych, 2001). Opioid receptor antagonism blocks estrogen-induced internalization; the proposed mechanism is that estrogen regulates the release of MOR-active endogenous peptides, one of which is β-END.

Central injections of NPY inhibit sexual receptivity and proceptivity in steroid-primed rodents. The first report of NPY in

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rats used a supraphysiological dose of estradiol benzoate (EB; 100 μg) to induce lordosis (Clark et al., 1985), but a subsequent study repeated their findings with a more physiological relevant dose of EB (5 μg), albeit in Syrian hamsters (Corp et al., 2001). Despite these results, the NPY receptor subtype mediating behavioral actions has not been elucidated; the NPY-Y_1 subtype (Y_1R) is responsible for NPY-induced luteinizing hormone (LH) release (Kalra et al., 1992; Besecke et al., 1994; Leupen et al., 1997). This suggested that Y_1R might be involved in reproduction. Additional evidence for this was the distribution of Y_1R in the ARH (Migita et al., 2001) and its regulation by sex steroids. Hypothalamic Y_1R mRNA levels are elevated on the afternoon of proestrus (but see Parker et al., 1996; Jain et al., 1999; Xu et al., 2000), indicating that Y_1R expression is regulated by sex steroids.

We hypothesized that the site of NPY action on endogenous opioid peptides may be the ARH, which is enriched in Y_1R immunoreactive cells and NPY and is the origin of β-END cell bodies that project and innervate the MPN. Moreover, in males, NPY immunoreactive fibers have been reported in close apposition to β-END immunoreactive neurons (Fuxe et al., 1997, 1998), which express the Y_1R subtype (Broberger et al., 1997; Pickel et al., 1998). These results suggest that NPY may activate Y_1R on β-END neurons, which in turn produce the initial inhibition of sexual receptivity after estradiol treatment of ovariectomized (OVX) rats.

The present study used behavioral analysis, immunocytochemistry, and retrograde tract tracing to determine whether the estradiol and progesterone regulation of reproductive behaviors was mediated by the activation of Y_1R in the ARH and activation of MORs in the MPN. These results have been presented in preliminary form (Mills et al., 2001).

Materials and Methods

Animal treatments. Adult female Long–Evans rats (200–255 gm; Charles River, Wilmington, MA) were OVX by the supplier and allowed to survive 2 weeks before experimentation. At the University of California, Los Angeles (UCLA), all animals were maintained in Plexiglas cages under controlled light (12 hr light/dark cycle) and temperature (22–24°C) with *ad libitum* access to food and water. All of the procedures were approved by the Chancellor's Animal Research Committee at UCLA.

Two weeks after being OVX, all animals were anesthetized with isoflourane (2–3% 1:1 oxygen) and placed in a stereotaxic frame, and a trephine was performed to implant a guide cannula (Plastics One, Roanoke, VA) into the third ventricle (3V). The coordinates used were anteroposterior, bregma (−0.8 mm), lateral (on the midline, above the superior longitudinal sinus), and dosoventral (−6.5 mm), and were defined by Swanson (1998). Beginning 3–5 d after surgery, rats were given a subcutaneous injection of EB (50 or 2 μg) or vehicle (Safflower oil), followed 30 hr later with an intracerebroventricular injection of vehicle [artificial CSF (aCSF); $n = 8$ per group], NPY (0.5 nmol; $n = 6$ per group; Bachem, Torrance, CA), Y_1R antagonist (Cys^{31},Nva^{34})-Neuropeptide Y (27–36)$_2$ ($CNNY_{27–36}$; 10 nmol; $n = 6$ per group; Bachem, King of Prussia, PA), or a selective Y_1R agonist, [Leu31, Pro34]-Neuropeptide Y (LPNY; 10 nmol; Bachem) through a 29 gauge inner cannula connected to a 25 μl Hamilton syringe via a plastic tube. The inner cannula extended 1 mm beyond the tip of the guide cannula. The duration of the injections was 1 min. To allow for diffusion, the inner cannula was left in place for an additional minute after microinjection. Thirty minutes after intracerebroventricular injection of drugs, rats were anesthetized deeply with sodium pentobarbital (100 mg/kg) and perfused transcardially with physiologic saline (4°C), followed by 4% paraformaldehyde in 0.1 M Sörensen's phosphate buffer (PB) (4°C). Brains were removed, postfixed for 4–24 hr in the paraformaldehyde solution, and then transferred to 15% sucrose in 0.1 M PB, pH 7.5, for cryoprotection.

To assess the level of receptor internalization after steroid treatment and to determine the interactions between steroids and NPY, animals were given injections of 2 μg of EB. Beginning 26 hr after EB injection, all animals were given 500 μg of progesterone. Four hours later, rats were given microinjections of vehicle (aCSF; $n = 6$) or NPY (10 nmol; $n = 8$) into the 3V, anesthetized, perfused, and processed for Y_1R and MOR immunocytochemistry.

Female receptive behavior. To determine whether activation of Y_1R in the ARH influences MOR in the MPN that ultimately modulates lordosis, OVX rats with a 3V guide cannula were given injections of 2 μg of EB and, 26 hr later, 500 μg of progesterone. At 30 hr after estrogen injection, rats received either a 3V microinjection of aCSF vehicle, NPY (0.5 nmol), a selective Y_1R agonist, LPNY (10 nmol; Bachem), or a combination of aCSF vehicle ($n = 6$) or D-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr amide (CTOP; 50 nmol/2 μl; $n = 6$; Bachem), followed 10 min later with an infusion of the selective Y_1R agonist LPNY (10 nmol) through a 29 gauge cannula.

Animals were tested for receptive (lordosis) behavior with a stimulus male at 15, 30, and 45 min after microinjection. A lordosis quotient (LQ; the percentage of lordotic postures the female displays × 100) was determined. The individual scoring lordosis was blind to the treatment of the animal. The LQ was analyzed using repeated measures ANOVA to assess main effects of time and treatment including any interaction (SigmaStat, version 2.11; Jandel Scientific, San Rafael, CA). If a significant main effect was obtained, *post hoc* comparisons were conducted using Student–Newman–Keuls test. $p < 0.05$ was considered significant.

Retrograde tract tracing experiments. OVX rats were implanted with a guide cannula aimed at the MPN. The coordinates used were anteroposterior, bregma (+0.1 mm), lateral (−0.8), and dosoventral (−6.1 mm) and were defined by Swanson (1998). A 29 gauge cannula containing the retrograde tracer FluoroGold (FG; 2% w/v; Fluorochrome, Denver, CO) extended 1 mm beyond the tip of the guide cannula into the MPN. One to 2 μl of FG was slowly ejected over 10 min from the cannula connected to a 25 μl Hamilton syringe via a plastic tube. The cannula was allowed to remain in place for an additional 10 min, and the animal was allowed to survive for 10–12 d ($n = 6$).

Immunocytochemisty. The distribution of Y_1R immunoreactivity was determined using a rabbit primary antibody raised against aa 356–382 of the rat Y_1R (DiaSorin, Stillwater, MN). Free-floating tissue sections were incubated in 4% NGS (Jackson ImmunoResearch Laboratories, West Cove, PA) in PBS for 60 min, followed by incubation with Y_1R antibody (1:750 diluted in 1% NGS and PBS) at 4°C for 18–48 hr. The sections were washed, incubated in blocking buffer (Tyramide Signal Amplification kit; NEN Life Science Products, Boston, MA) for 30 min, and then incubated in biotin-conjugated goat anti-rabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 hr. Tissue sections were washed in 0.1 M TBS, pH 7.5, incubated in streptavidin–HRP (1:100; NEN Life Science Products) for 30 min, washed in TBS with Tween 20 (Sigma, St. Louis, MO), incubated for 5 min in Flouroscein-conjugated tyramide (1:50; Tyramide Signal Amplification kit; NEN Life Science Products), and then washed in 0.1 M Tris buffer without saline, pH 7.5, and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Mounted sections were air-dried and coverslipped using Vectashield mounting medium (Vector Laboratories).

The MOR antibody was raised in rabbits against a synthetic fragment (LENLEAETAPLP) corresponding to the intracellular C terminus of rat MOR ($MOR_{387–398}$; a gift from C. Evans and B. Anton, UCLA, Los Angeles, CA). Free-floating tissue sections were washed in PBS and then incubated in 1% NGS, 1% BSA, and 0.5% Triton X-100 in PBS for 60 min. Tissue sections were incubated for 48–72 hr at 4°C with the affinity-purified MOR antibody (1:5000) diluted in PBS with 1% NGS and 0.17% Triton X-100. The sections were washed, incubated with a goat anti-rabbit secondary antibody, and processed as described previously.

β-END was localized with rabbit primary antibodies generated against a synthetic ovine β-END bound to keyhole limpet hemocyanin (ICN Biomedicals, Costa Mesa, CA). Immunocytochemistry and localization of β-END was performed by incubating the tissue in PBS with 0.3% Triton X-100, 20% NGS, and β-END antiserum (1:10,000) at 4°C for 18–48 hr. After the β-END antiserum, tissue was washed, incubated with a goat anti-rabbit antibody, and processed as described above. Tetramethylrhodamine-conjugated tyramide (1:50; Tyramide Signal

Amplification kit; NEN Life Science Products) was used for visualization of the antibody antigen sites of interest.

To establish Y_1R and β-END immunoreactive colocalization in the ARH, free-floating tissue sections were processed for β-END immunocytochemistry, washed in TBS, and avidin/biotin-binding sites were blocked by incubating sections in avidin D (1:100 in TBS) for 15 min, followed by biotin (1:100 in TBS) for 15 min (Vector Laboratories). Tissue sections were then processed for Y_1R immunocytochemistry.

Tissue sections from each treatment were grouped and processed in parallel to avoid procedural artifacts and to ensure consistency. Immunocytochemical controls included the omission of primary and secondary antisera and preabsorption of antibodies with appropriate peptide epitopes. Omission of primary antibodies and preabsorption with appropriate epitopes blocked all staining. For the double-label studies, the pattern of staining was compared with the pattern in singly labeled sections, the order of primary antibodies was reversed, and heterologous absorption controls (e.g., the Y_1R antiserum was absorbed with β-END peptide) were done. The pattern of staining for each antibody was the same. Neither heterologous preabsorption of primary antibodies nor the order of primary antibodies affected the staining.

Image analysis. All immunocytochemical-labeled sections were analyzed with a Zeiss Axioskop 2 equipped with epiflouresecent illumination, Axiocam CCD camera, and digital image analysis system (Zeiss USA, Thornwood, NY). To identify internalization, tissue sections were examined with a LSM 410 laser-scanning confocal microscope system (Zeiss USA) to visualize the subcellular distribution of Y_1R, β-END, and MOR immunoreactivity. The excitation source was a krypton–argon laser (Coherent, Santa Clara, CA) with output at 488, 568, and 633 nm. Fluorescein was imaged with a 488 nm emission filter and a 515–540 nm bandpass filter. Rhodamine was imaged with a 530 nm emission filter and 560–610 nm bandpass filter. Images were adjusted for brightness and contrast using the LSM-PC (Zeiss USA) and Adobe PhotoShop (version 6.0) programs before printing with an Epson 1200 color printer.

Y_1R and MOR immunoreactivity were considered internalized when the majority of immunoreactivity was observed as puncta within the cytoplasm of neuronal cell bodies and processes (Mantyh et al., 1995; Eckersell et al., 1998; Sinchak and Micevych, 2001). An increased level of internalization was correlated with an increased number of immunoreactive structures (Dournaud et al., 1998; Eckersell et al., 1998; Sinchak and Micevych, 2001, 2003). Therefore, to obtain an estimate of the relative internalization, the area of Y_1R immunoreactivity in the ARH and MOR immunoreactivity in the dorsal aspect of the medial part of the MPN in every fourth or fifth section was estimated using the KS-300 imaging software (version 3.0; Zeiss USA). Briefly, at a magnification of 225×, threshold brightness was established that discriminated specific from nonspecific staining. This calibration was determined for each measured section. The percentage of area occupied by specific staining (i.e., staining above the threshold) was determined and compared between the treatment groups. Statistical analysis was done separately for Y_1R immunoreactivity in the ARH and MOR immunoreactivity in the MPN. Treatment groups were compared using a one-way ANOVA. When a significant main effect was obtained, differences were analyzed further using a Student–Newman–Keuls *post hoc* test (SigmaStat, version 2.11; Jandel Scientific).

Quantitative measurements of Y_1R/β-END and Y_1R/β-END/FG coexistence were performed by counting the number of ARH cell bodies immunoreactive for β-END and Y_1R alone and doubly labeled with β-END and Y_1R immunoreactivity. To determine the population of ARH neurons projecting to the MPN, FG, FG/Y_1R, FG/β-END immunoreactivity and FG/Y_1R/β-END immunoreactivity were counted along the rostrocaudal axis of the ARH. To prevent double counting of cells, new counts were adjusted using the Abercrombie correction (Abercrombie, 1946). The total numbers of single-labeled, double-labeled, or triple-labeled cells were averaged across animals.

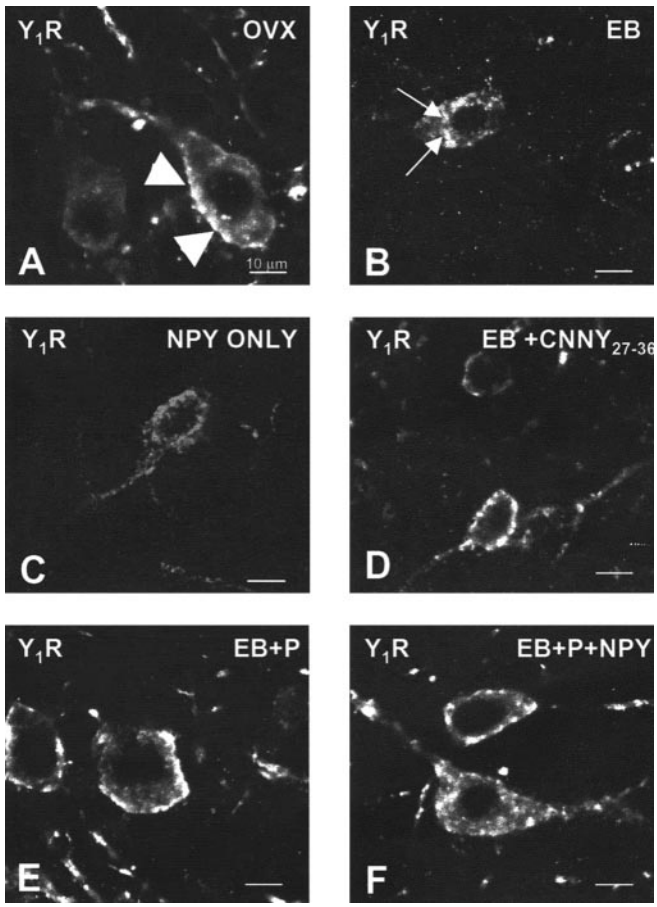

Figure 1. Confocal projections of Y_1R neurons in the ARH of OVX (*A*), EB (*B*), NPY (*C*), $CNNY_{27–36}$ (*D*), EB plus progesterone (EB + P; *E*), or EB + P + NPY-treated (*F*) rats. Large arrowheads indicate Y_1R immunoreactivity associated with the plasma membrane (*A*, *D*, *E*), suggesting that these receptors have not been activated. Small arrows indicate the immunoreactivity was not associated with the plasma membrane, suggesting an internalization of Y_1R (*B*, *C*, *F*). Please note that OVX females showed Y_1R immunoreactivity associated with the membrane and in the cytoplasm.

Results

Experiment 1: Do treatments that induce Y_1R internalization also internalize MOR?

Within the mediobasal hypothalamus, the distribution of Y_1R is most pronounced along the third ventricle, in the periventricular nucleus, and in the ARH (data not shown). We observed the greatest number of Y_1R immunoreactive cells in the ARH, as reported previously (data not shown) (Mikkelsen and Larsen, 1992; Migita et al., 2001). Medial and lateral parts of the ARH contained Y_1R immunoreactivity that was distributed in a ventral band beneath the ventromedial nucleus (see below). Y_1R immunoreactivity decorated cell bodies and processes of ARH neurons. β-END immunoreactive staining was generally present in the cytoplasm of large ARH neurons (see below).

In the ARH of OVX rats, Y_1R antibodies outlined neuronal cell bodies and processes, suggesting that the Y_1R is associated with the plasma membrane (Fig. 1*A*). Some cytoplasmic puncta were also observed in Y_1R immunoreactive cells, suggesting that a portion of Y_1R were internalized in the estrogen-free condition. Treatment with either 50 or 2 μg of EB produced a similar reorganization of Y_1R immunoreactivity. The majority of the immunoreactivity was observed as cytoplasmic punta, whereas the membrane-associated localization of Y_1R immunoreactivity was

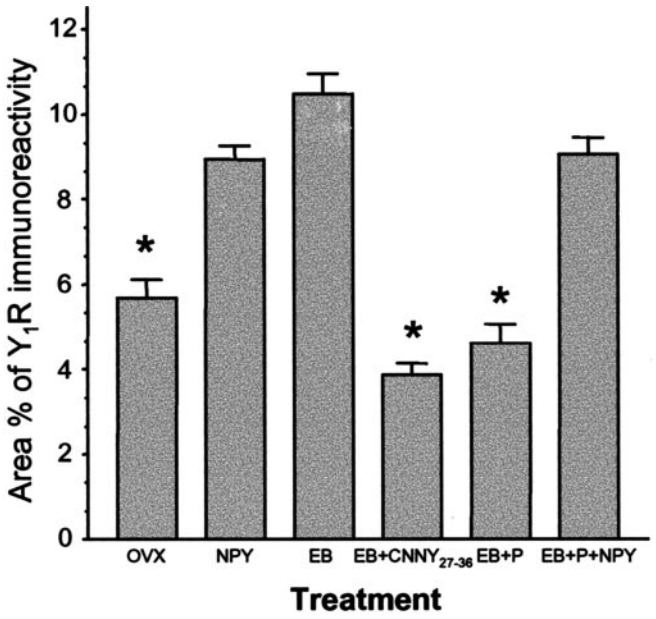

Figure 2. Percentage area of Y_1R in the ARH of OVX rats treated with vehicle (OVX), NPY, EB, EB plus Y_1R antagonist (EB + $CNNY_{27–36}$), EB plus progesterone (EB + P), or EB + P + NPY. Values presented are means ± SEM. *$p < 0.05$ versus NPY, EB, and EB + P + NPY.

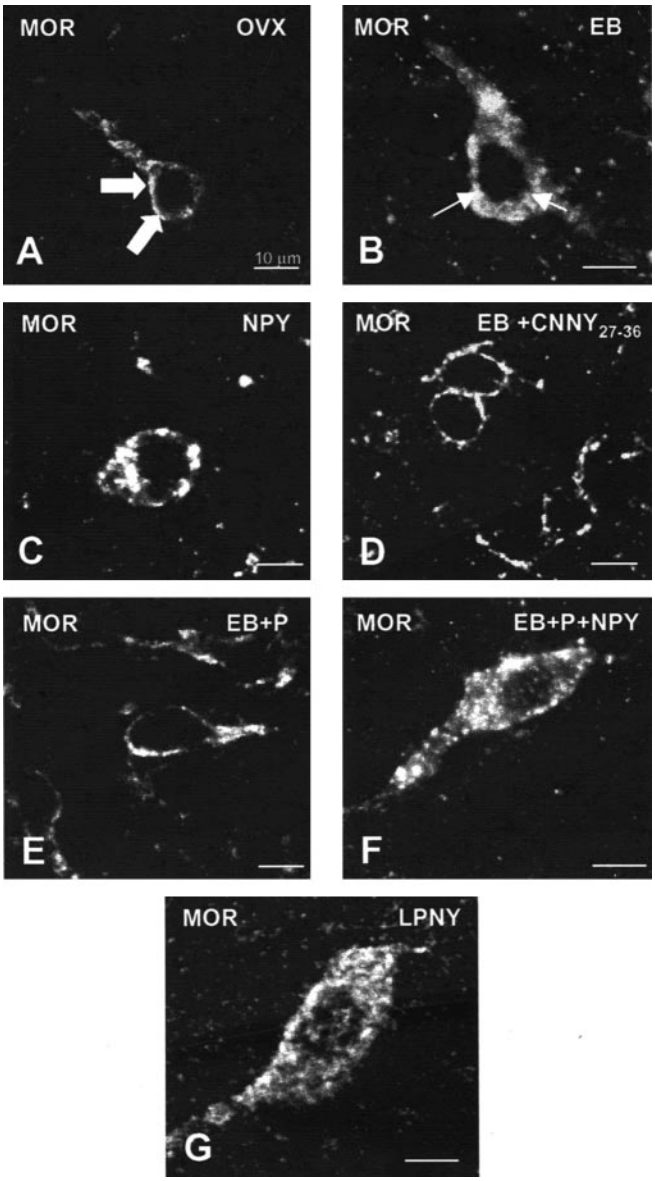

Figure 3. Confocal projections of MOR immunoreactive neurons and processes in the MPN of OVX (*A*), NPY (*B*), EB (*C*), $CNNY_{27–36}$ (*D*), EB plus progesterone (EB + P; *E*), EB + P + NPY-treated (*F*) rats or LPNY. Large arrowheads indicate MOR immunoreactivity associated with the plasma membrane (*A*, *D*, *E*), suggesting that these receptors have not been activated. Small arrows indicate that immunoreactivity was not associated with the plasma membrane, suggesting an internalization of the MOR (*B*, *C*, *F*, *G*).

reduced substantially (Fig. 1*B*). A significantly greater amount of Y_1R immunoreactive cell bodies and processes was associated with an internalized pattern of staining in EB-treated females compared with OVX animals (Fig. 2). Within the MPN, β-END immunoreactive fibers were distributed in the periventricular preoptic area and the medial MPN (Cheung and Hammer, 1995), overlapping the distribution of MOR immunoreactivity (Micevych et al., 1997; Eckersell et al., 1998; Sinchak and Micevych, 2001). In the MPN of OVX rats, MOR immunoreactivity was associated with the plasma membrane (Fig. 3*A*). Steroid treatments altered the subcellular distribution of MOR immunoreactivity and the amount of β-END immunoreactivity but not the tissue distribution. As we reported previously, EB-induced MOR internalization was also observed in the MPN (Fig. 3*B*) (Eckersell et al., 1998; Sinchak and Micevych, 2001). Similarly, there was a significant increase of MOR immunoreactivity in EB-treated females compared with OVX animals (Fig. 4).

Infusion of NPY (0.5 nmol) into the 3V of OVX rats induced internalization of Y_1Rs in the ARH (Fig. 1*C*) and MORs in the MPN (Fig. 3*C*). The infusion of NPY also corresponded to increased Y_1R and MOR immunoreactive cell bodies and processes compared with the OVX group (Figs. 2 and 4). This internalization was similar to that seen after treatment with EB (Figs. 2 and 4), suggesting that activation of Y_1R in the ARH may be related to EB- or NPY-induced MOR internalization. Indeed, LPNY (Y_1R-specific agonist) infused into the 3V of OVX females induced internalization of MORs in the MPN (Fig. 3*G*). Y_1R immunoreactivity was not internalized in $CNNY_{27–36}$ (Y_1R antagonist)-treated rats given EB (Fig. 1*D*). Similar to the Y_1R result, $CNNY_{27–36}$ also blocked MOR internalization in the MPN (Fig. 3*D*). There was no significant change in MPN MOR immunoreactivity and ARH Y_1R fiber density between $CNNY_{27–36}$ and OVX animals but a significant decrease in MPN MOR immunoreactivity and Y_1R fiber density between EB plus $CNNY_{27–36}$-treated animals, and EB treatment was observed ($p < 0.05$) (Figs. 2 and 4).

Experiment 2: Is progesterone blockade of EB-induced MOR internalization mediated through the Y_1R?

As described above, EB induced Y_1R and MOR internalization, however, Y_1R immunoreactivity in the ARH and MOR immunoreactivity in the MPN were associated with the plasma membrane and not internalized in EB plus progesterone-treated OVX rats (Figs. 1*E*, 3*E*). The progesterone blockade of MOR internalization was superceded by the 3V infusion of NPY, which internalized Y_1R in the ARH and MOR in the MPN (Figs. 1*F*, 3*F*). Quantified, the internalization was significantly increased in Y_1R and MOR labeling compared with vehicle treatment ($p < 0.05$) (Figs. 2 and 4).

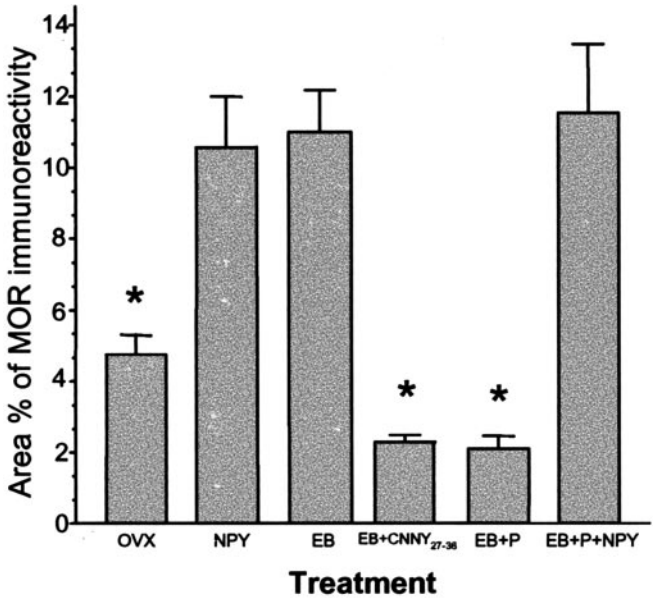

Figure 4. Area percentage of neuropeptide MOR immunoreactivity in the MPN was measured in female rats treated with vehicle (OVX), NPY, EB, EB plus Y_1R antagonist (EB+$CNNY_{27–36}$), EB plus progesterone (EB+P), or EB+P+NPY. Values presented are means ± SEM. *$p < 0.05$ versus NPY, EB, and EB+P+NPY.

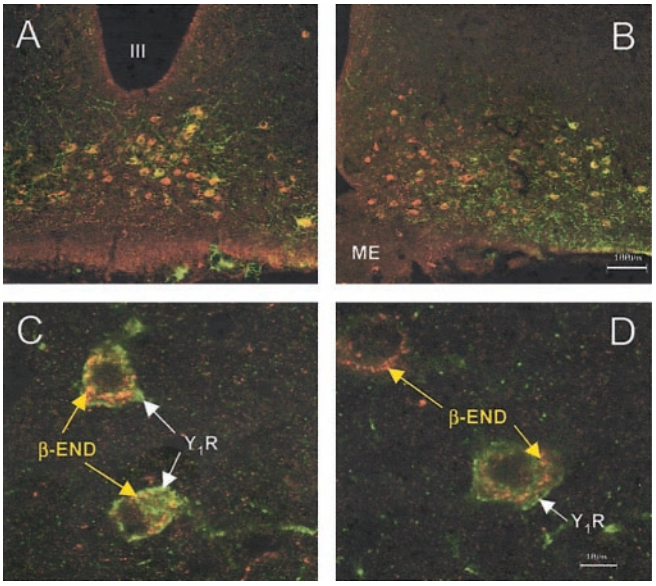

Figure 5. Confocal projections of β-END and Y_1R immunoreactivity in the ARH and doubly labeled neurons of estrogen-primed female rats. *A*, *B*, Low-power magnification of the distribution of Y_1R neurons labeled with FITC (green) and β-END somata labeled with tetramethylrhodamine (TRITC; red). β-END immunoreactivity decorated structures within the cytoplasm. The Y_1R immunoreactivity was associated with the plasma membrane. Although most Y_1R immunoreactive neurons were β-END immunoreactive, only a subpopulation of β-END immunoreactive neurons was Y_1R immunoreactive (43%). *C*, *D*, Higher-power magnification of doubly labeled Y_1R/β-END and β-END neurons in the ARH. Note that the Y_1R immunoreactivity is associated with the plasma membrane, whereas the β-END immunoreactivity labels punctuate structures in the cell bodies.

Experiment 3: Do β-END immunoreactive neurons have Y_1R immunoreactivity?

The distribution of Y_1R and β-END cell bodies overlapped in the ARH (Fig. 5). To determine whether Y_1R and β-END cell bodies were colocalized, sections containing the ARH were stained with antibodies directed against Y_1R and β-END. Most of the Y_1R immunoreactive neurons were immunoreactive for β-END (95 ± 1.5%). Almost half of the β-END immunoreactive neurons were immunoreactive for Y_1R (43 ± 2.6%). No significant change of the percentage of colocalization was measured among the various steroid or peptide treatments ($p > 0.05$).

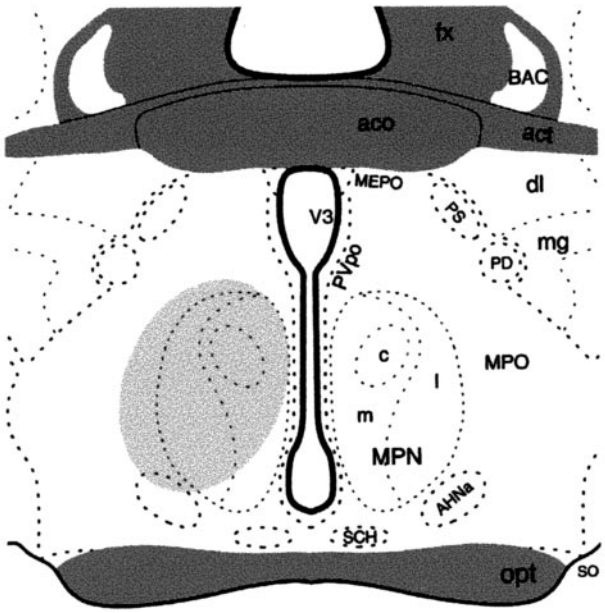

Figure 6. A schematic drawing of the medial preoptic (MPO) area through the anterior hypothalamus showing the extension of the FG injection site as indicated by the shaded portion. AHNa, Anterior hypothalamic nucleus, anterior part; act, anterior commissure, temporal limb; aco, anterior commissure; BAC, bed nucleus anterior commissure; dl, bed nuclei stria terminalis, anterior division, dorsolateral nucleus; fx, fornix; mg, bed nuclei stria terminalis, anterior division, magnocellular nucleus; LPO, lateral preoptic area; MEPO, median preoptic nucleus; MPNl, medial preoptic nucleus, lateral part; MPNm, medial preoptic nucleus, medial part; MPNc, medial preoptic nucleus, central part; opt, optic chiasm; PD, posterodorsal preoptic nucleus; PS, parastrial nucleus; Pvpo, preoptic periventricular nucleus; SCH, suprachiasmatic nucleus; SO, supraoptic nucleus; V3, third ventricle. (Adapted from Swanson, 1998).

Experiment 4: identification of ARH neurons projecting to MPN

To label MPN efferents from the ARH, the retrograde tracer FG was microinjected into the MPN. Animals that were analyzed for this experiment had injection sites centered on the MPN (Fig. 6). Injection of FG into the MPN resulted in dense retrograde labeling throughout the rostrocaudal axis of the ARH. At all levels of the ARH, retrograde-labeled cells were distributed between Y_1R and β-END immunoreactive cells. A population, 30 ± 0.02% of the retrogradely labeled ARH cells, was Y_1R and β-END immunoreactive (Fig. 7). In addition, 12 ± 0.03% of the FG-labeled cells were Y_1R immunoreactive, and another 28 ± 0.05% were β-END immunoreactive.

Experiment 5: specific Y_1R activation inhibited lordosis

To correlate receptive behavior with steroid-induced receptor trafficking, OVX rats were treated with 2 μg of EB plus 500 μg of progesterone every 4 d to induce sexual receptivity. In these steroid-primed females, injection of LPNY resulted in a significant overall decrease in lordosis compared with the vehicle controls ($F_{(1,44)} = 79.08$; $p < 0.05$). A significant interaction was noted between treatment group (LPNY and vehicle control) and time (15, 30, and 45 min after injection) of LQ scores ($F_{(2,44)} =$

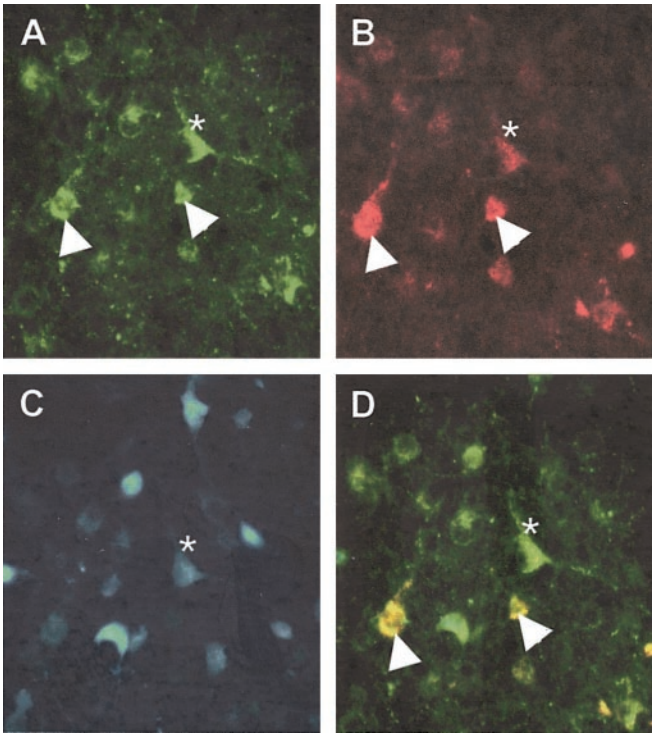

Figure 7. Photomicrograph of retrogradely labeled cells from the ARH after injection of FG into the MPN. The same field imaged to excite the FITC labeling Y_1R (*A*), tetramethylrhodamine (TRITC) labeling β-END (*B*), and FG-labeling efferent projections (*C*) from the MPN. The images are combined in *D*. Doubly labeled β-END and Y_1R immunoreactivity are yellow (arrowheads), and triple-labeled are white (asterisk).

7.68; $p < 0.05$). *Post hoc* analysis reveled that LPNY significantly decreased lordosis after 15, 30, and 45 min compared with vehicle-injected rats (Fig. 8*B*) ($p < 0.05$), indicating a correlation between neurochemical events demonstrating activation of the Y_1R and MOR and lordosis behavior. There was neither a significant change in lordosis in the vehicle control across time nor when using NPY (Fig. 8*A*) ($p > 0.05$).

Experiment 6: specific MOR blockade restores lordosis after Y_1R activation

To determine whether MOR activation was downstream of Y_1R circuits, EB plus progesterone-primed females received intracerebroventricular microinjections of CTOP (50 nmol/2 μl) or vehicle (2 μl aCSF), followed 10 min later with LPNY (10 nmol/μl). There was a significant difference between CTOP-infused females and vehicle control ($F_{(1,32)} = 48.15$; $p < 0.05$) and significant interaction between treatment group (CTOP or aCSF) and time (15, 30, or 45 min after injection) of LQ scores ($F_{(2,32)} = 5.35$; $p < 0.05$). *Post hoc* analysis revealed that the CTOP plus Y_1R agonist-microinjected females showed a significantly greater LQ score at 15, 30, and 45 min when compared with aCSF plus Y_1R (Fig. 8C) ($p < 0.05$). This indicates that CTOP administered before LPNY blocked the inhibitory effects of the Y_1R agonist on lordosis.

Discussion

The major finding of these experiments is that activation of the Y_1R in the mediobasal hypothalamus mediated by estrogen results in activation of MOR in the MPN that regulates sexual receptivity. Several lines of evidence support this finding. First, intracerebroventricular NPY or a selective Y_1R agonist (LPNY) into OVX rats induced the activation/internalization of MOR in the MPN, which was similar to the activation/internalization of MOR in the MPN after estrogen treatment (Eckersell et al., 1998; Sinchak and Micevych, 2001). Second, the estrogen-induced

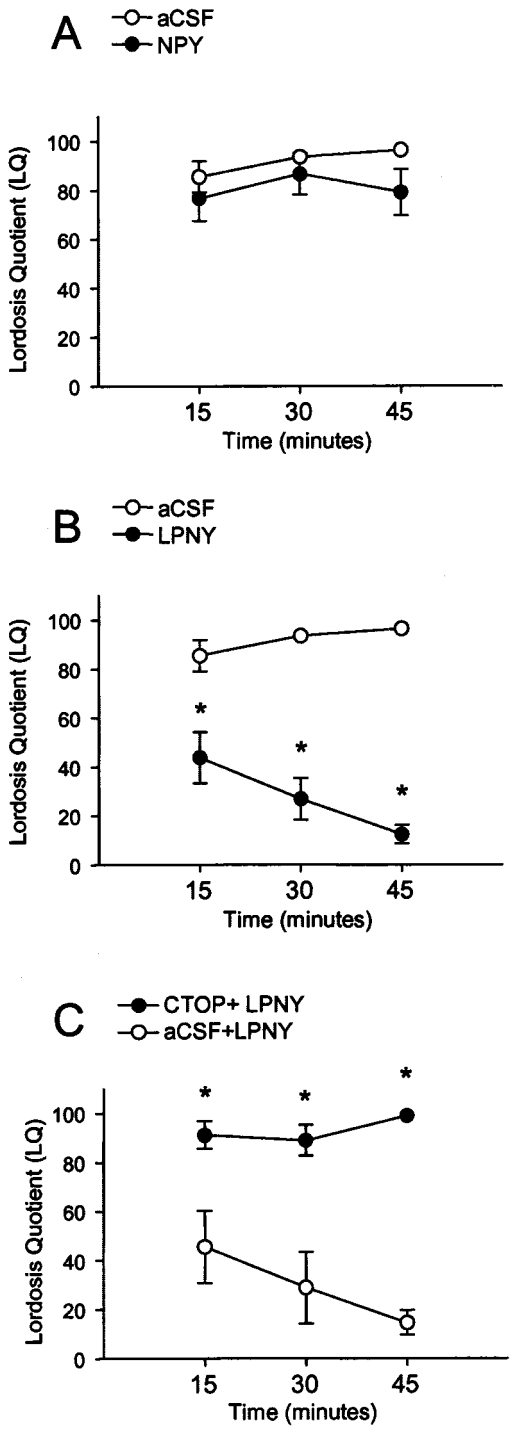

Figure 8. Sexual receptivity measured by LQ in 2 μg of EB plus 500 μg of progesterone (P)-primed female rats infused with NPY or vehicle (aCSF) (*A*); LPNY, a neuropeptide Y-Y_1 receptor agonist, or aCSF (*B*); or CTOP plus LPNY or aCSF+LPNY (*C*) into the 3V (intracerebroventricular). LQ was measured 15, 30, and 45 min after intracerebroventricular injection. There was significant decrease in lordosis in Y_1R agonist-injected females compared with aCSF controls (*B*), whereas CTOP+LPNY (*C*) prevented the inhibition of lordosis at 15, 30, and 45 min after microinjection into the 3V. There was no significant difference in NPY-treated animals (*A*). *$p < 0.05$ versus aCSF.

MOR internalization/activation was blocked by $CNNY_{27–36}$, a selective antagonist of the Y_1R. Third, progesterone that blocked the estrogen-induced activation/internalization of MOR was reversed by NPY, indicating that the Y_1R mediates progesterone actions that block estrogen-induced MOR internalization in the MPN. Fourth, internalization or noninternalization was correlated with behavior. EB plus progesterone facilitated lordosis, and LPNY, the Y_1R agonist, inhibited lordosis. In addition, the MOR-specific antagonist CTOP reversed the Y_1R-induced inhibition of lordosis. The present results combined with previous studies demonstrated that steroid activation of the Y_1R and MOR in the hypothalamus inhibited lordosis (Sirinathsinghji, 1984; Clark et al., 1985; Gorzalka et al., 1997; Torii et al., 1999; Corp et al., 2001; Sinchak and Micevych, 2001). Finally, the present results provide evidence that these peptidergic circuits are not redundant but are organized in series to regulate behavior.

Although MOR activation in the MPN is correlated with inhibition of lordosis, the specific endogenous opioid peptide involved in the activation remains to be elucidated. Several types of endogenous opioid peptides activate the MOR, including β-END, the enkephalins, and the endomorphins (Borsodi and Toth, 1995). δ-Opioid receptors preferentially bind enkephalin, but β-END and the endomorphins preferentially bind to MOR and inhibit EB or EB plus progesterone-induced lordosis (Sirinathsinghji, 1984; Sinchak and Micevych, 2001). Enkephalin and β-END expression have been shown to be regulated by estrogen (Wilcox and Roberts, 1985; Priest et al., 1995; Holland et al., 1998). Although endomorphins are the most MOR-selective endogenous opioid peptides (Zadina et al., 1999), to date, no studies indicate a steroid or NPY regulation of endormorphin cells. In contrast, estrogen-receptive β-END cell bodies are located in the ARH (Morrell et al., 1985), a region enriched in NPY and Y_1R (Finley et al., 1981).

In the present study, we demonstrated that in female rats nearly all Y_1R immunoreactive cells were β-END immunoreactive, a result that is consistent with previous findings in male rats (Fuxe et al., 1997). Approximately 43% of the β-END immunoreactivity were Y_1R immunoreactive, and approximately one-third of the β-END/Y_1R immunoreactive neurons project to the MPN, demonstrating that a significant population of β-END/Y_1R immunoreactive neurons project to the MPN (Cheung et al., 1995). The present findings integrate reports that MOR inhibition of lordosis is mediated by the MPN (Sirinathsinghji, 1986; Torii and Kubo, 1994; Cheung et al., 1995; Sinchak and Micevych, 2001) and that NPY inhibition is mediated by the mediobasal hypothalamus (Clark et al., 1985).

In addition to localizing the Y_1R in the ARH, our results demonstrate that activation of a specific receptor is necessary for NPY inhibition of lordosis. In our hands, central injections of NPY did not inhibit lordosis; however, selective activation of the Y_1R with LPNY inhibited lordosis. These results are congruent with the finding that Y_1R mediates NPY actions on LH release (Kalra et al., 1992; Leupen et al., 1997; Li et al., 1999). The present results extend the observation that NPY inhibited lordosis in rats with a very robust estrogen-priming paradigm (100 μg of EB) (Clark et al., 1985). In our study, NPY did not inhibit lordosis in 2 μg of EB plus 500 μg of progesterone-treated rats; however, specific activation of Y_1R did inhibit lordosis. We correlated the activation of this receptor (internalization) in the ARH with the inhibition of lordosis. One possibility for NPY for the discrepancy was that lordosis induced by EB is different from that induced by EB plus progesterone, as reported by Mani (2001).

Our results demonstrate the utility of using receptor internalization to map the activation of circuits in the CNS (Mantyh et al., 1995; Micevych et al., 1997). The phenomenon of receptor internalization is nearly ubiquitous. G-protein-coupled receptors, growth factor receptors, and, more recently, glutamate receptors, undergo internalization after agonist activation (Zaki et al., 2000; Mundell et al., 2001; Belcheva and Coscia, 2002). Although, in most cases, the internalization is related to desensitization and downregulation, it has become clear that internalization also may be involved in signaling (Vieira et al., 1996; Luttrell et al., 1997; Daaka et al., 1998; Ignatova et al., 1999). To our knowledge, we are the first to report that estrogen-induced MOR activation/internalization in the MPN is dependent on Y_1R activation/internalization in the ARH. Treatment of OVX rats with EB or NPY that induced Y_1R internalization also induced MOR internalization, whereas EB-primed animals treated with a Y_1R antagonist, $CNNY_{27–36}$, blocked MOR internalization. Similarly, treatments that prevented EB-induced MOR internalization, such as progesterone, were overcome by treatment with NPY. Thus, MOR internalization, which is related to the EB-induced inhibition of lordosis, requires activation of Y_1R.

The site of estrogen activation remains to be elucidated. Estrogen has been shown to regulate the expression of β-END and its precursor mRNA (proopiomelanocortin) in the ARH (Barden et al., 1981; Wardlaw et al., 1982; Wilcox et al., 1986; Priest and Roberts, 2000). However, only a small population of β-END cells have estrogen receptors (Morrell et al., 1985; Jirikowski et al., 1986), suggesting that the observed estrogen effect is indirect, via transsynaptic mechanisms. To date, there is one preliminary report showing that a majority of NPY neurons have estrogen receptors (Cator et al., 2002). Estrogen receptor-α mRNA expression has been reported in the ARH (Shughrue et al., 1997; Osterlund et al., 1998), suggesting the possibility that estrogen directly regulates NPY synthesis and release. Our current results document Y_1R on β-END neurons in the female rat ARH. When taken together with reports that NPY expression is regulated by estrogen (Crowley et al., 1985; Sahu et al., 1992; Baskin et al., 1995), our results suggest that gonadal steroid information is transduced through NPY neurons to β-END neurons that project to the MPN (Cheung et al., 1995; Cheung and Hammer, 1995), in which infusion of β-END antiserum augments estrogen-induced lordosis (Sirinathsinghji, 1986).

In summary, the present experiments support the hypothesis that estradiol and progesterone actions on NPY that sequentially activate Y_1R and then MOR are part of the lordosis regulating circuitry that includes ARH β-END neurons that project to the MPN. These steroid-activated neuropeptide circuits are sequentially activated and functionally interrelated to modulate sexual receptivity and reproduction.

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