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Dual Label *in Situ* Hybridization Studies Provide Evidence that Luteinizing Hormone-Releasing Hormone Neurons Do Not Synthesize Messenger Ribonucleic Acid for μ, κ, or δ Opiate Receptors*

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ABSTRACT

Abundant evidence suggests that opiatergic neurons play an important intermediary role in the regulation of LHRH release by ovarian steroids; however, it is unclear whether opiates communicate directly or indirectly with LHRH neurons. To investigate this issue, we used dual label *in situ* hybridization histochemistry to determine whether LHRH neurons synthesize messenger RNA (mRNA) for μ, κ, and/or δ opiate receptors. For these studies, we examined both intact (n = 3) and ovariectomized, steroid-treated rats. Ten of the ovariectomized rats were implanted 1 week later (day 0) with SILASTIC brand (Dow Corning) capsules of estradiol. On the morning of day 2, half of the estradiol-treated rats were injected with 5 mg progesterone. All animals were killed at approximately 1530 h on day 2. We found that cells expressing μ, κ, and δ opiate receptor mRNAs were in all sections that also contained LHRH neurons. In every case, LHRH neurons were seen to be surrounded by or in close proximity to cells containing μ, κ, or δ mRNAs. However, regardless of steroid treatment, we found no neurons containing both LHRH mRNA and mRNAs encoding any of the three receptor subtypes. These results support the hypothesis that LHRH neurons are regulated indirectly by opiatergic neurons. (*Endocrinology* **138:** 1667–1672, 1997)

IT IS KNOWN that ovarian steroids exert both negative and positive feedback effects on the synthesis (1, 2) and release of LHRH (3); however, it is still not clear how LHRH neurons receive steroid signals. Previous studies showed that few, if any, LHRH neurons have classical estrogen (4, 5) or progesterone (6) receptors. Therefore, changes in the levels of steroids must be communicated to LHRH neurons by other neuropeptide or neurotransmitter systems or through a nonclassical interaction with receptors of these systems (7, 8). Available evidence suggests that endogenous opiates and their receptors may comprise one of the systems that translates ovarian hormone signals into changes in LHRH and LH release (reviewed in Ref. 9).

The results of numerous studies indicate that opiates are inhibitory to LH secretion. For example, it has been shown that the opiate receptor antagonist, naloxone, blocks the negative feedback effects of estradiol (E_2) (10) and increases LHRH (11) and LH (12, 13) release. Furthermore, the opiate agonist, morphine, blocks ovulation (14) by interfering with the positive feedback effects of ovarian steroid on LH (15) and LHRH release (16). These findings suggest that endogenous opiates inhibit LHRH neurons and that escape from this inhibition is an important component of the estrogen-dependent trigger for LHRH and LH surge release.

Although there is considerable evidence that opiatergic neurons regulate LHRH release, it remains controversial whether this regulation is direct or indirect. A number of studies have shown that an opioid-noradrenergic interaction in the preoptic area (POA) is an important part of the neural circuitry controlling LH release (9, 17). Opiate antagonists stimulate LH release and concomitantly increase noradrenergic activity (18), whereas morphine blocks both estrogen-induced increases in noradrenergic activity and the LH surge (18, 19). Other studies suggest that a decrease in the release of excitatory amino acids may also mediate the inhibitory effects of opiates on LH release (20). Thus, there is convincing evidence that opiates affect LH release indirectly by inhibiting the release of stimulatory neurotransmitters.

However, there are also data suggesting that LHRH neurons are regulated directly by opiatergic neurons. The results of several neuroanatomical studies indicate that β-endorphinergic neurons synapse on LHRH neurons in rats (21, 22) as well as in juvenile monkeys (23). Furthermore, GT1–1 cells, hypothalamically derived transformed tumor cells with many characteristics of LHRH neurons (24), appear to have binding sites for δ receptor-specific ligands (25). Thus, the neurocircuitry through which opiates modulate LHRH release remains uncertain.

To resolve the question of whether LHRH neurons are regulated directly or indirectly by opiate neurons, it is important to determine what opiate receptor subtype mediates the effects of opiates on LHRH and LH release. The μ opiate receptor appears to be the major subtype involved in regulating steroid-dependent LH release (26, 27). However, μ, κ, and δ receptor antagonists can all increase LH release in ovariectomized (OVX), estrogen-treated rats (27). Likewise, agonists for each of these opiate receptor subtypes suppress

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the secretion of LH in OVX rats (28, 29). Thus, it is presently unclear whether one or several opiate receptor subtypes mediate the effects of ovarian steroids on LHRH and LH secretion.

We reasoned that if LHRH neurons are regulated directly by any of the endogenous opiates, they must synthesize receptors for the μ, κ, or δ opiate receptor. Therefore, in the present studies, we used dual label *in situ* hybridization histochemistry (ISHH) to determine whether LHRH messenger RNA (mRNA) and mRNA encoding μ, κ, and δ opiate receptors are colocalized in neurons of the POA. We chose dual label ISHH for these studies rather than dual label immunocytochemistry primarily because it is easier to verify colocalization of LHRH and receptor mRNAs than the corresponding proteins. The mRNA encoding LHRH and various receptors generally resides in cell bodies or dendrites, whereas receptor proteins are commonly found in the axon terminal regions of complex neuropil (30), where it is difficult to determine colocalization with certainty. For these studies, we examined animals that were OVX, OVX and treated with E_2, or OVX and treated with E_2 and progesterone to control for the possibility that the steroid milieu alters the expression of opiate receptor mRNAs.

Materials and Methods

Animals

All animals used in these studies were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols used were approved by the committee on animal care at the University of Massachusetts. Female adult Sprague-Dawley rats, weighing 225–250 g (Charles River, Wilmington, MA), were maintained under controlled temperature and photoperiod conditions (14 h of light, 10 h of darkness; lights on, 0500 h) with food and water available *ad libitum*.

For study 1, animals (n = 3) were intact. For studies 2A and B, animals were OVX (n = 15) under metofane anesthesia (methoxyfluor, Pitman Moore, Mundelein, IL) and given steroid replacement as described previously (31, 32). One week after ovariectomy (day 0), some animals (n = 10) were sc implanted with SILASTIC brand capsules (Dow Corning, Midland, MI; id, 1.57 mm; od, 3.8 mm; length, 30 mm) containing E_2 (150 μg/ml sesame oil). On day 2, half of the E_2-treated animals (n = 5) were given a sc injection of progesterone in oil (5 mg/rat) at 0900 h. All animals were killed at 1530 h. Brains were rapidly removed, frozen on dry ice, wrapped in parafilm, and stored in tightly capped conical tubes at −80 C until cryosectioned.

Study 1

For this study, randomly cycling female rats (n = 3) were killed at 0900 h. Frozen coronal sections (12 μm) were collected from the region of the diagonal band of Broca through the anterior hypothalamus (A 7890-A 6670) (33). Sections were thaw-mounted to gelatin-coated microscope slides and stored at −80 C until they were used for hybridization. Adjacent sections were hybridized with radiolabeled complementary RNA (cRNA) probes specific for μ, κ, or δ opiate receptor mRNAs and digoxigenin-labeled cRNA probes for LHRH mRNA. For this study, radiolabeled probes were prepared by *in vitro* transcription in the presence of 90 pmol [^{35}S]UTP and 30 pmol UTP as described below. Tissue preparation, hybridization, and detection methods are also described below.

Study 2

In study 1, we observed that μ, κ, and δ receptor mRNAs were present in highest abundance in the region containing the organum vasculosum of the lamina terminalis (OVLT) and the rostral POA (rPOA; A 7470-A 6790) (33). In addition, this region contained most of the LHRH neurons. Therefore, in studies 2A and B, we obtained 12-μm cryosections from the rPOA/OVLT region and stored them as described for study 1. For study 2A, sets of three adjacent sections from OVX and OVX steroid-treated rats described above were simultaneously hybridized to digoxigenin-labeled cRNA probes for LHRH mRNA and to ^{35}S-labeled cRNA probes for μ, κ, and δ opiate receptors. However, in this study probes were labeled to a higher specific activity than those used in study 1. These probes were prepared by *in vitro* transcription in the presence of 120 pmol [^{35}S]UTP and no unlabeled UTP. For study 2B, a second set of adjacent sections from the OVX and OVX steroid-treated rats was hybridized to probes prepared with 120 pmol [^{33}P]UTP and no unlabeled UTP.

Tissue preparation

On the day of hybridization, sections were warmed to room temperature for 10 min, fixed in 4% PBS-formalin for 15 min, treated with 0.25% acetic anhydride in 0.1 M triethanolamine-0.9% sodium chloride (pH 8.0), dehydrated and delipidated in a series of ethanol and chloroform washes, and rehydrated with 95% ethanol. Tissue sections were then air-dried and hybridized to probes prepared as described below.

Probe preparation

Radiolabeled cRNA probes for μ, κ, and δ opiate receptor mRNAs. A 450-bp *Bam*HI-*Xba* fragment corresponding to the 3′-untranslated region of the μ opiate receptor mRNA was linearized using *Apa*I (34). A 773-bp fragment (corresponding to nucleotides 1351–2124 of the κ opiate receptor mRNA that encode 45 bp of the terminal coding region and the 3′-untranslated region) was linearized with *Hin*dIII (35, 36). A 983-bp *Hin*dIII-*Sst*I fragment (corresponding to nucleotides 304-1287 of the δ opiate receptor mRNA that encode the first extracellular loop to transmembrane domain VII) was linearized using *Pst*I (36, 37). The specificity of the μ and δ opiate receptor cRNA probes was previously verified by Northern blot analysis (37, 38). The κ opiate receptor clone was used previously to map the distribution of κ opiate receptor mRNA with *in situ* hybridization in the rat central nervous system (36).

Radiolabeled cRNA probes for the opiate receptor mRNAs were prepared by drying down either 90 (study 1) or 120 (study 2A) pmol [^{35}S]UTP (DuPont-New England Nuclear, Boston, MA) or 120 pmol [^{33}P]UTP (study 2B; DuPont-New England Nuclear) in a DNA Speed-Vac (Savant, Farmingdale, NY). One microgram of linearized template; 5 × transcription buffer; 100 mM dithiothreitol (DTT); 20 U RNAsin; 0.5 mM ATP, CTP, and GTP; either 30 pmol (study 1) or no (studies 2A and 2B) UTP; and 10 U of the appropriate RNA polymerase were added. The μ opiate receptor complementary DNA (cDNA) was transcribed with SP6 polymerase, the κ opiate receptor cDNA with T3 polymerase, and the δ opiate receptor cDNA with T7 polymerase. The transcription mixture was incubated for 30 min at 37 C, then a second aliquot of RNA polymerase (10 U) was added and incubated again for 30 min at 37 C. The template was degraded with 2 U deoxyribonuclease in the presence of 20 U RNAsin, 1 M Tris-HCl, 1 M $MgCl_2$, and 0.5 μl transfer RNA (25 μg/μl). The radiolabeled cRNA probes were extracted with phenol-chloroform, then precipitated twice with NaCl and ethanol and resuspended in 100 μl 1 mM EDTA and 10 mM Tris.

Digoxigenin-labeled LHRH cRNA probe. A 330-bp *Bam*HI-*Hin*dIII cDNA fragment that corresponds to exons I–IV of LHRH mRNA (39) was linearized with *Hin*dIII. Digoxigenin-UTP-labeled cRNA probes were transcribed using 1 μg linearized LHRH cDNA template, T7 RNA polymerase (20 U; Promega, Madison, WI), 5 × transcription buffer, 0.5 mM ATP, CTP, and GTP, 0.1 mM UTP, 0.5 mM digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN), 100 mM DTT, and 20 U RNAsin. This mixture was incubated at 37 C for 1 h, then an additional 20 U polymerase were added, and the mixture was incubated for another hour at 37 C. Deoxyribonuclease (2 U), in the presence of 20 U RNAsin, was then used to degrade the DNA template. The probe was precipitated with NaCl and ethanol, and resuspended in a solution of 100 μl 1 mM EDTA and 10 mM Tris. The specificity of this probe for LHRH mRNA has been previously verified by Northern blot analysis (40).

Hybridization and probe detection

For these studies, dual labeled ISHH was performed as described previously (41). For all studies, each section was hybridized with 25 μl

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hybridization buffer containing 500,000 cpm/section of the desired radiolabeled opiate receptor cRNA probe and 1 μl digoxigenin-labeled LHRH probe. The concentration of radiolabeled probe was based on preliminary studies of the signal to background ratio for various concentrations of ^{35}S- and ^{33}P-labeled probes. For subsequent hybridizations, we chose the concentration above which there was no further increase in signal. The hybridization buffer contained 2 × SSC (standard saline citrate); 50% (vol/vol) formamide; 10% dextran sulfate (wt/vol); 250 μg/ml transfer RNA; 500 μg/ml sheared, single stranded, salmon sperm DNA; 1 × Denhardt's solution (0.002% Ficoll, 0.002% polyvinylpyrrolidone, and 0.02% BSA); and 50 mM DTT. Sections were hybridized overnight at 55 C under glass coverslips. After hybridization, coverslips were removed in 1 × SSC at room temperature. Sections were washed for 5 min in 1 × SSC at room temperature, incubated for 5 min, then 20 min in 2 × SSC-50% formamide at 52 C, followed by two 1-min washes in 2 × SSC at room temperature. Sections were incubated in ribonuclease buffer consisting of 100 μg/ml ribonuclease A (Boehringer Mannheim), 5 M NaCl, 1 M Tris, and 0.5 M EDTA; washed three times for 5 min each time in 2 × SSC at room temperature; incubated for 5 min in 2 × SSC-50% formamide at 52 C; and washed twice for 5 min each time in 2 × SSC. After the last wash, sections were processed for immunocytochemical detection of the digoxigenin-labeled probes. In studies 1 and 2A, sections were blocked with 2% BSA (Boehringer Mannheim) in buffer A (100 mM Tris and 150 mM NaCl) with 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO) for 1 h. The sections were rinsed twice for 5 min each time in buffer A, then incubated in antidigoxigenin-peroxidase (antidig-POD; 1:200 in 2% BSA in buffer A) for 48 h at 4 C. After incubation, the sections were rinsed twice for 5 min each time in buffer A. They were then incubated for approximately 20 min in 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma). The DAB solution was prepared by mixing 10 mg DAB in 50 ml 0.1 M Tris (pH 7.6), filtering the solution through Whatman paper (no. 1, Clifton, NJ), and adding 8 μl hydrogen peroxide. The sections were rinsed twice in 0.1 M Tris (pH 7.6) for 5 min each time, dipped quickly in deionized distilled water, and then rinsed for 3 min in 70% ethanol.

In study 2B, we changed blocking agents because preliminary studies showed that 5% blocking reagent (Boehringer Mannheim) yielded lower nonspecific binding than 2% BSA in buffer A. Therefore, tissue sections in study 2B were blocked with 5% blocking reagent in 0.1 M sodium maleate (pH 7.5) with 0.3% Triton X-100 for 1 h at room temperature. The sections were next rinsed twice in 0.1 M sodium maleate for 5 min each time at room temperature, then incubated for 48 h at 4 C in antidig-POD diluted 1:200 in 2% blocking reagent in 0.1 M sodium maleate. After incubation, the sections were rinsed twice for 5 min each time in 0.1 M sodium maleate. They were then incubated for 4 h in DAB solution as described above. Finally, sections were rinsed twice in 0.1 M Tris, pH 7.6, for 5 min each time, dipped quickly in deionized distilled water, and rinsed for 3 min in 70% ethanol.

After the digoxigenin-labeled probes were detected immunocytochemically, the radiolabeled probes were visualized autoradiographically by dipping the slides in Kodak NTB3 (Eastman Kodak, Rochester, NY) diluted 1:1 with deionized distilled water. Slides were exposed for 2–3 weeks at 4 C, then developed in Kodak Dektol, and fixed in Kodak fixer. Slides were examined microscopically using a ×40 objective to determine the number of LHRH neurons that were and were not dual labeled.

Results

In study 1, dual label ISHH of sections from cycling rats showed no evidence of colocalization of LHRH mRNA with mRNAs encoding any of the opiate receptor mRNAs in 136 LHRH neurons examined (see Table 1). Likewise, in studies 2A and B, dual label ISHH of sections from OVX steroid-treated rats showed no evidence of colocalization in 785 LHRH neurons, even though probes used for these studies were of higher specific activity (transcribed in the presence of 100% either [^{35}S]UTP or [^{33}P]UTP) than in study 1. Replacement of BSA with blocking reagent in study 2B reduced the background signal of the digoxigenin-labeled probes, but did not affect the results of the dual label ISHH. The results of these studies are summarized in Table 1.

In study 1, we found that in the brain sections containing LHRH neurons, μ and κ opiate receptor mRNAs were each present in highest abundance in the region containing the rPOA/OVLT, specifically in the region lateral to and above the optic recess and OVLT (nucleus preopticus, pars suprachiasmatica; A 7470) (33). Cells expressing δ receptor mRNAs were also present in this region, but were not as numerous as those expressing μ or κ opiate receptor mRNAs. Based on the findings of study 1, we focused on the rPOA/OVLT in study 2. Although we saw no evidence of colocalization, we observed that LHRH neurons were always surrounded by or in close proximity to cells that expressed μ, κ, or δ receptor mRNA. Figure 1 shows representative sections of the dual label ISHH studies for LHRH mRNA and μ, δ, or κ receptor mRNA in the nucleus preopticus, pars suprachiasmatica.

Discussion

The present findings support the hypothesis that opiates regulate LHRH neurons indirectly. We found no evidence of μ, κ, or δ opiate receptor mRNA in LHRH neurons despite the abundance of each of these mRNAs in cells surrounding LHRH neurons. Neither OVX nor steroid treatment affected colocalization of LHRH and opiate receptor mRNAs. Thus,

TABLE 1. Dual label *in situ* hybridization studies to determine whether μ, δ, and/or κ opiate receptor mRNA was expressed in LHRH neurons in the rostral preoptic area of random cycling, OVX, OVX with E_2 treatment, and OVX with E_2 and P treatment animals

Treatment	Opiate receptor probe	LHRH neurons examined	Dual labeled LHRH neurons
Random cycling (n = 3)	μ	70	0
Random cycling (n = 3)	δ	25	0
Random cycling (n = 3)	κ	41	0
OVX (n = 5)	μ	42	0
OVX + E_2 (n = 5)	μ	80	0
OVX + E_2 + P (n = 5)	μ	123	0
OVX (n = 5)	δ	88	0
OVX + E_2 (n = 5)	δ	133	0
OVX + E_2 + P (n = 5)	δ	68	0
OVX (n = 5)	κ	110	0
OVX + E_2 (n = 5)	κ	75	0
OVX + E_2 + P (n = 5)	κ	66	0
Total		921	0

n, Number of animals examined per steroid treatment.

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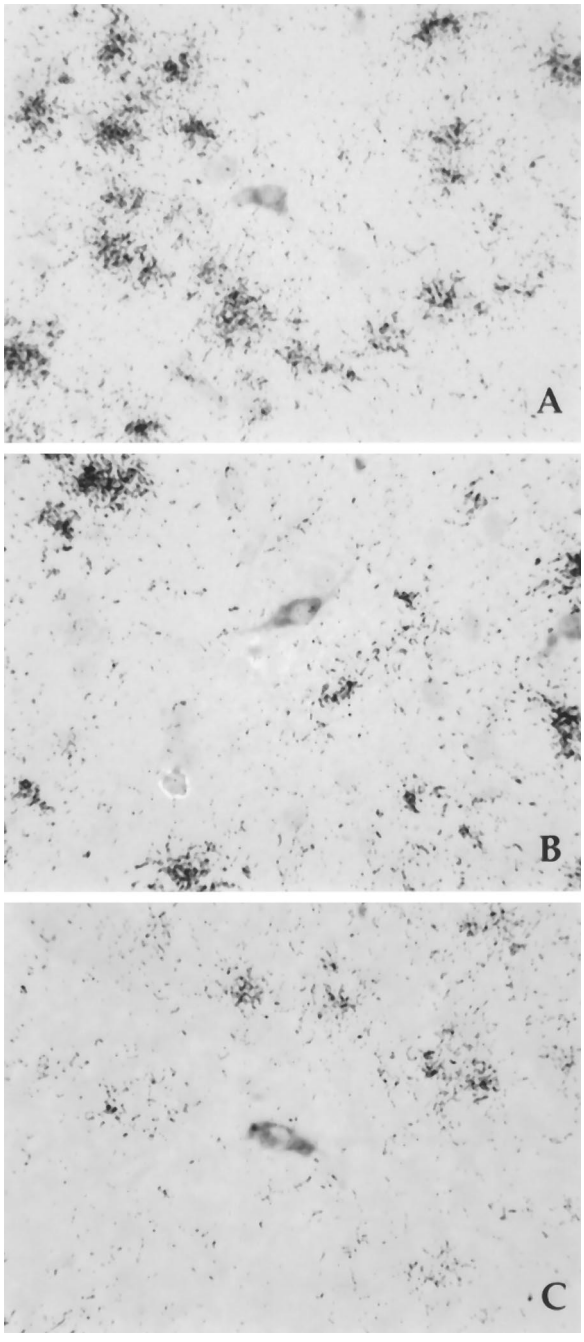

FIG. 1. Photomicrographs showing the results of dual label ISHH studies detecting LHRH mRNA with a digoxigenin-labeled cRNA probe and μ (A), κ (B), or δ (C) opiate receptor mRNAs with ^{35}S-labeled cRNA probes. These were 12-μm sections from the region of the rPOA/OVLT. Although there are numerous cells expressing opiate receptor mRNAs, there is no evidence of cellular colocalization of LHRH mRNA and mRNA for any of the opiate receptors.

it seems likely that opiates regulate LHRH neurons indirectly through other steroid-sensitive neuronal systems.

Our findings suggest that LHRH neurons are not directly regulated by opiates. These results contrast with those of previous studies showing that ACTH- or β-endorphin-immunoreactive terminals form synaptic contacts with LHRH neurons in female rats (21), male rats (22), and juvenile monkeys (23). Furthermore, Lagrange *et al.* (42) showed that in guinea pig brain, the selective μ-agonist, DAMGO, hyperpolarizes hypothalamic LHRH neurons in the presence of tetrodotoxin. These results suggest that LHRH neurons are regulated by β-endorphinergic neurons through a μ opiate receptor. However, recent studies of LHRH-producing GT1–1 cells indicate that these cells do not have μ opiate-binding sites (25), nor is LHRH release from these cells affected by μ opiate receptor agonists (43). Rather, pharmacological studies suggest that LHRH release from GT1 cells is regulated by opiates through a δ receptor (25, 43). Our findings do not reconcile the results of the studies described above, but instead, indicate that LHRH neurons in the female rat brain do not appear to synthesize mRNA for either μ or δ receptors.

It is possible that the discrepancy between our data and data suggesting that LHRH neurons are directly regulated by opiates may be attributable to the sensitivity of the ISHH method. We addressed this issue by using cRNA probes labeled to high specific activity by transcribing the cDNA templates in the presence of [^{33}P]UTP with no competing UTP. Although these probes readily detected the mRNAs for μ, κ, and δ opiate receptors in cells around LHRH neurons, we cannot rule out the possibility that levels of these mRNAs are extremely low in LHRH neurons and below the limits of detectability of our assay. Furthermore, it is possible that these receptor mRNAs are expressed only under a specific endocrine milieu not replicated by our steroid treatments, or that they are expressed for a only a short period of time during the estrous cycle.

Previous studies on the distribution of opiate receptor mRNAs did not examine all regions that contain LHRH neruons. However, similar to our findings, Delfs *et al.* (38), Mansour *et al.* (36), and Minami *et al.* (44) reported moderate to intense expression of μ opiate receptor mRNA in the medial POA (mPOA). Also consistent with our findings, κ receptor mRNA was observed previously in the mPOA (36, 45, 46). δ Receptor mRNA was observed in lower abundance than κ and μ and was seen in only scattered cells of the mPOA (36). Our studies extend previous work by showing that the highest abundance of μ, κ, and δ opiate receptor mRNAs are in the rPOA/OVLT, a region that includes the anteroventral periventricular nucleus (Pe_{AV}) and the median preoptic nucleus.

Although the possibility that LHRH neurons express very low levels of opiate receptor mRNAs cannot be ruled out, our findings are more consistent with the idea that opiates regulate LHRH neurons indirectly, possibly through the opiate receptor-containing cells residing in the rPOA/OVLT region. For example, although the Pe_{AV} and median preoptic nucleus are regions of the rPOA/OVLT region that do not contain LHRH neurons, they are important for estrogen-dependent LH surge release. The Pe_{AV} has a high concentration of estrogen receptor-containing (47) and estrogen receptor mRNA-containing neurons (48). Interestingly, we found previously that antiestrogen implants into this region block estrogen-dependent LH surge release (1, 49) and that estrogen induces the greatest increase in LHRH mRNA before the LH surge in neurons of this region (2). Furthermore, electrolytic lesions of structures in the periventricular region

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of the POA block the preovulatory LH surge and ovulation (50, 51). Finally, the PE_{AV} contains sexually dimorphic populations of opiatergic neurons that are regulated differently in males and females (52). Thus, it seems possible that the estrogen signal for ovulation is communicated at least in part to LHRH neurons by the rPOA/OVLT cells, in which we observed high levels of μ, κ, and/or δ opiate receptor mRNAs.

Although we do not currently know the identity of the opiate receptor mRNA-expressing cells in the rPOA/OVLT region, it seems likely that they are stimulatory neurons. In support of this idea, there is evidence that the inhibitory effects of β-endorphin may result from a decrease in the release of excitatory amino acids (20). This possibility is particularly interesting in view of recent findings that a subpopulation of LHRH neurons (53) as well as GT1 cell lines (54, 55) express glutamate receptor subtype mRNAs. Thus, it is possible that at least some of the opiate receptor-containing cells in the rPOA are glutaminergic and that opiatergic neurons regulate LHRH neurons indirectly by interfering with the release of this stimulatory neurotransmitter.

It is also possible that the effects of opiates on LHRH and LH release are indirectly mediated by other stimulatory neurotransmitters whose cell bodies reside outside the rPOA/OVLT, possibly noradrenergic and adrenergic neurons. This idea is supported by findings suggesting that opiates act presynaptically to decrease the release of catecholamines. For example, the stimulatory effects of the opiate receptor antagonist, naloxone, on LH release are prevented by interference with the synthesis or binding of epinephrine and norephinephrine (56–58). Moreover, the magnitude of LH release induced by naloxone depends upon the activity of noradrenergic neurons at the time of treatment (19). Naloxone also increases hypothalamic catecholamine turnover rates (18) and concurrently increases catecholamine and LHRH release from perifused hypothalamic fragments (59). In contrast, morphine blocks both the estrogen-dependent afternoon surge in LH release and the increase in norepinephrine turnover rates that normally accompany the surge (60). Finally, androgenization abolishes the positive feedback effects of estrogen on LH release and alters noradrenergic responses to naloxone (61, 62).

In conclusion, neurons of the rPOA/OVLT that express LHRH mRNA do not express mRNA encoding μ, κ, or δ opiate receptor. These results support the hypothesis that LHRH neurons are indirectly regulated by endogenous opiates. Further studies will be required to determine the phenotypic identity of the cells of the rostral POA that do express opiate receptor mRNA and to identify the neurocircuitry through which opiates regulate LHRH and LH release.

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References

1. **Petersen SL, Cheuk C, Hartman RD, Barraclough CA** 1989 Medial preoptic microimplants of the antiestrogen, Keoxifene, affect luteinizing hormone-releasing hormone mRNA levels, median eminence luteinizing hormone-releasing hormone concentrations and luteinizing hormone release in ovariectomized, estrogen-treated rats. J Neuroendocrinol 1:279–289
2. **Petersen SL, McCrone S, Keller M, Shores S** 1995 Effects of estrogen and progesterone on luteinizing hormone-releasing hormone messenger ribonucleic acid levels: considerations of temporal and neuroanatomical variables. Endocrinology 136:3604–3610
3. **Levine JE, Ramirez VD** 1982 Luteinizing hormone-releasing hormone release during the rat estrous cycle and after ovariectomy as estimated with push-pull cannulae. Endocrinology 111:1439–1448
4. **Shivers BD, Harlan RE, Morrell JI, Pfaff DW** 1983 Absence of oestradiol concentration in cell nuclei of LHRH immunoreactive neurons. Nature 304:345–347
5. **Watson REJ** 1992 Further evidence that most luteinizing hormone-releasing hormone neurons are not directly estrogen-responsive: simultaneous localization of luteinizing hormone-releasing hormone and estrogen receptor immunoreactivity in the guinea-pig brain. J Neuroendocrinol 4:311–317
6. **Fox SR, Harlan RE, Shivers BD, Pfaff DW** 1990 Chemical characterization of neuroendocrine target for progesterone in the female rat brain and pituitary. Neuroendocrinology 51:276–283
7. **Ramirez VD, Dluzen DE, Ke FC** 1990 Effects of progesterone and its metabolites on neuronal membranes. Ciba Found Symp 153:125–141
8. **McEwen BS** 1991 Non-genomic and genomic effects of steroids on neural activity. Trends Pharmacol Sci 12:141–147
9. **Kalra SP** 1993 Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge. Endocr Rev 14:507–538
10. **Van Vugt DA, Sylvester PW, Aylsworth CF, Meites J** 1982 Counteraction of gonadal steroid inhibition of luteinizing hormone release by naloxone. Neuroendocrinology 34:274–278
11. **Karahalios DG, Levine JE** 1988 Naloxone stimulation of *in vivo* LHRH release is not diminished following ovariectomy. Neuroendocrinology 47:504–510
12. **Bruni JF, Van Vugt DA, Marshall S, Meites J** 1977 Effects of naloxone, morphine and methionine enkephalin on serum prolactin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone, and growth hormone. Life Sci 21:461–466
13. **Ieiri T, Chen HT, Meites J** 1980 Effects of naloxone and morphine on the proestrous surge of prolactin and gonadotropins in the rat. Endocrinology 106:1568–1570
14. **Barraclough CA, Sawyer CH** 1955 Inhibition of the release of pituitary ovulatory hormone in the rat by morphine. Endocrinology 57:329–337
15. **Pang CN, Zimmerman E, Sawyer CH** 1977 Morphine inhibition of the preovulatory surges of plasma luteinizing hormone and follicle stimulating hormone in the rat. Endocrinology 101:1726–1732
16. **Ching M** 1983 Morphine suppresses the proestrous surge of GnRH in pituitary portal plasma of rats. Endocrinology 112:2209–2211
17. **Dyer RG, Grossmann R, Mansfield S, Diez-Guerra FJ, Bicknell RJ, Hollingsworth S** 1988 Opioid peptides inhibit noradrenergic transmission in the preoptic area to block LH secretion: evidence from neonatally androgenised rats. Brain Res Bull 20:721–727
18. **Adler BA, Crowley WR** 1984 Modulation of luteinizing hormone release and catecholamine activity by opiates in the female rat. Neuroendocrinology 38:248–253
19. **Akabori A, Barraclough CA** 1986 Effects of morphine on luteinizing hormone secretion and catecholamine turnover in the hypothalamus of estrogen-treated rats. Brain Res 362:221–226
20. **Bonavera JJ, Kalra SP, Kalra PS** 1993 Evidence that luteinizing hormone suppression in response to inhibitory neuropeptides, β-endorphin, interleukin-1β, and neuropeptide-K, may involve excitatory amino acids. Endocrinology 133:178–182
21. **Leranth C, MacLusky NJ, Shanabrough M, Naftolin F** 1988 Immunohistochemical evidence for synaptic connections between pro-opiomelanocortin-immunoreactive axons and LH-RH neurons in the preoptic area of the rat. Brain Res 449:167–176
22. **Chen W-P, Witkin JW, Silverman AJ** 1989 Beta-endorphin and gonadotropin-releasing hormone synaptic input to gonadotropin-releasing hormone neurosecretory cells in the male rat. J Comp Neurol 286:85–95
23. **Thind KK, Goldsmith PC** 1988 Infundibular gonadotropin releasing hormone neurons are inhibited by direct opioid and autoregulatory synapses in juvenile monkeys. Neuroendocrinology 47:203–216
24. **Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI** 1990 Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5:1–10
25. **Maggi R, Pimpinelli F, Martini L, Piva F** 1995 Characterization of functional opioid δ receptors in a luteinizing hormone-releasing hormone-producing neuronal cell line. Endocrinology 136:289–295
26. **Pfeiffer DG, Pfeiffer A, Shimohigashi Y, Merriam GR, Loriaux DL** 1983 Predominant involvement of μ- rather than δ- or κ-opiate receptors in LH secretion. Peptides 4:647–649
27. **Panerai AE, Petraglia F, Sacerdote P, Genazzani AR** 1985 Mainly μ-opiate receptors are involved in luteinizing hormone and prolactin secretion. Endocrinology 117:1096–1099
28. **Leadem CA, Kalra SP** 1985 Effects of endogenous opioid peptides and opiates on luteinizing hormone and prolactin secretion in ovariectomized rats. Neuroendocrinology 41:342–352
29. **Leadem CA, Yagenova SV** 1987 Effects of specific activation of μ-, δ-, and

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κ-opioid receptors on the secretion of luteinizing hormone and prolactin in the ovariectomized rat. Neuroendocrinology 45:109–117
30. **Elde R, Schalling M, Ceccatelli S, Nakanishi S, Hokfelt T** 1990 Localization of neuropeptide receptor mRNA in rat brain: initial observations using probes for neurotensin and substance P receptors. Neurosci Lett 120:134–139
31. **Wise PM, Camp-Grossman P, Barraclough CA** 1981 Effects of estradiol and progesterone on plasma gonadotropins, prolactin and LHRH in specific brain areas of ovariectomized rats. Biol Reprod 24:820–830
32. **Petersen SL, Keller ML, Carder SA, McCrone S** 1993 Differential effects of estrogen and progesterone on levels of POMC mRNA levels in the arcuate nucleus: relationship to the timing of LH surge release. J Neuroendocrinol 5:643–648
33. **Konig JFR, Klippel RA** 1967 The Rat Brain. A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem. Krieger, New York
34. **Chen Y, Mestek A, Liu J, Hurley JA, Yu L** 1993 Molecular cloning and functional expression of a μ-opioid receptor from rat brain. Mol Pharmacol 44:8–12
35. **Meng F, Xie G-X, Thompson RC, Mansour A, Goldstein A, Watson SJ, Akil H** 1993 Cloning and pharmacological characterization of a rat κ opioid receptor. Proc Natl Acad Sci USA 90:9954–9958
36. **Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ** 1994 μ, δ, and κ opioid receptor mRNA expression in the rat CNS: an *in situ* hybridization study. J Comp Neurol 350:412–438
37. **Fukuda K, Kato S, Mori K, Nishi M, Takeshima H** 1993 Primary structures and expression from cDNAs of rat opioid receptor δ- and μ-subtypes. Fed Eur Biochem Soc 327:311–314
38. **Delfs JM, Kong H, Mestek A, Chen Y, Yu L, Reisine T, Chesselet M-F** 1994 Expression of μ opioid receptor mRNA in rat brain: an *in situ* hybridization study at the single cell level. J Comp Neurol 345:46–68
39. **Adelman JP, Mason AJ, Hayflick JS, Seeburg PH** 1986 Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin releasing-inhibiting factor in human and rat. Proc Natl Acad Sci USA 83:179–183
40. **Bond CT, Hayflick JS, Seeburg PH, Adelman JP** 1989 The rat gonadotropin-releasing hormone: SH locus: structure and hypothalamic expression. Mol Endocrinol 3:1257–1262
41. **Petersen SL, McCrone S** 1993 Use of dual-label in situ hybridization histochemistry to determine the receptor complement of specific neurons. In: Valentino KL, Eberwine JJ, Barchas JD (ed) In Situ Hybridization Applications to Neurobiology. Oxford University Press, New York, p 78
42. **Lagrange AH, Ronnekleiv OK, Kelly MJ** 1995 Estradiol-17β and μ-opioid peptides rapidly hyperpolarize GnRH neurons: a cellular mechanism of negative feedback? Endocrinology 135:2341–2344
43. **Maggi R, Pimpinelli F, Martini L, Piva F** 1995 Inhibition of luteinizing hormone-releasing hormone secretion by δ-opiod agonists in GT1–1 neuronal cells. Endocrinology 136:5177–5181
44. **Minami M, Onogi T, Toya T, Katao Y, Hosoi Y, Maekawa K, Katsumata S, Yabuuchi K, Satoh M** 1994 Molecular cloning and *in situ* hybridization histochemistry for rat μ-opioid receptor. Neurosci Res 18:315–322
45. **Minami M, Hosoi Y, Toya T, Katao Y, Maekawa K, Katsumata S, Yabuuchi K, Onogi T, Satoh M** 1994 In situ hybridization of κ-opioid receptor mRNA in the rat brain. Neurosci Lett 162:161–164
46. **Mansour A, Fox CA, Meng F, Akil H, Watson SJ** 1994 κ_1 receptor mRNA distribution in the rat CNS: Comparison to κ_1 receptor binding and prodynorphin mRNA. Mol Cell Neurosci 5:124–144
47. **Pfaff D, Keiner M** 1973 Atlas of estradiol-containing cells in the central nervous system of the female rat. J Comp Neurol 161:121–158
48. **Simerly RB, Chang C, Muramatsu M, Swanson LW** 1990 Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. J Comp Neurol 294:76–95
49. **Petersen SL, Barraclough CA** 1989 Suppression of spontaneous LH surges in estrogen-treated ovariectomized rats by microimplants of antiestrogen into the preoptic brain. Brain Res 484:279–289
50. **Wiegand SJ, Terasawa E** 1982 Discrete lesions reveal functional heterogeneity of suprachiasmatic structures in regulation of gonadotropin secretion in the female rat. Neuroendocrinology 34:395–404
51. **Ronnekleiv OK, Kelly MJ** 1986 Luteinizing hormone-releasing hormone neuronal system during the estrous cycle of the female rat: effects of surgically induced persistent estrus. Neuroendocrinology 43:564–576
52. **Gu G, Simerly RB** 1994 Hormonal regulation of opioid peptide neurons in the anteroventral periventricular nucleus. Horm Behav 28:503–511
53. **Eyigor O, Jennes L** 1996 Identification of glutamate receptor subtype mRNAs in gonadotropin-releasing hormone neurons in rat brain. Endocrine 4:133–139
54. **Mahachoklertwattana P, Sanchez J, Kaplan SL, Grumbach MM** 1994 *N*-Methyl-D-aspartate (NMDA) receptors mediate the release of gonadotropin-releasing hormone (GnRH) by NMDA in a hypothalamic GnRH neuronal cell line (GT1–1). Endocrinology 134:1023–1030
55. **Urbanski HF, Fahy MM, Daschel M, Meschul C** 1994 *N*-Methyl-D-aspartate receptor gene expression in the hamster hypothalamus and in immortalized luteinizing hormone-releasing hormone neurones. J Reprod Fertil 100:5–9
56. **Kalra SP, Simpkins JW** 1981 Evidence for noradrenergic mediation of opioid effects on luteinizing hormone secretion. Endocrinology 109:776–782
57. **Van Vugt DA, Aylsworth CF, Sylvester PW, Leung FC, Meites J** 1981 Evidence for hypothalamic noradrenergic involvement in naloxone-induced stimulation of luteinizing hormone release. Neuroendocrinology 33:261–264
58. **Kalra SP, Crowley WR** 1982 Epinephrine synthesis inhibitors block naloxone-induced LH release. Endocrinology 111:1403–1405
59. **Leadem CA, Crowley WR, Simpkins JW, Kalra SP** 1985 Effects of naloxone on catecholamine and LHRH release from perifused hypothalamus of the steroid-primed rat. Neuroendocrinology 40:497–500
60. **Akabori A, Barraclough CA** 1986 Effects of morphine on luteinizing hormone secretion and catecholamine turnover in the hypothalamus of estrogen-treated rats. Brain Res 362:221–226
61. **Grossmann R, Diez-Guerra FJ, Mansfield S, Dyer RG** 1987 Neonatal testosterone modifies LH secretion in the adult female rat by altering the opioid-noradrenergic interaction in the medial preoptic area. Brain Res 415:205–210
62. **Crowley WR** 1988 Sex differences in the responses of hypothalamic luteinizing hormone-releasing hormone and catecholamine systems to ovarian hormones and naloxone: implications for sexual differentiation of luteinizing hormone secretion in rats. Brain Res 461:314–321

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