Molecular Neurobiology

ISSN0893-7648/03/27(2): 197–222/$20.00

Visualizing Activation of Opioid Circuits by Internalization of G Protein-Coupled Receptors

Kevin Sinchak* and Paul Micevych

Department of Neurobiology, David Geffen School of Medicine at UCLA and the Laboratory of Neuroendocrinology of the Brain Research Institute UCLA, Los Angeles, CA 90095-1763

Abstract

Mu-opioid receptor (MOR) and opioid receptor-like receptor (ORL-1) circuits in the limbic hypothalamic system are important for the regulation of sexual receptivity in the female rat. Sexual receptivity is tightly regulated by the sequential release of estrogen and progesterone from the ovary suggesting ovarian steroids regulate the activity of these neuropeptide systems. Both MOR and ORL-1 distributions overlap with the distribution of estrogen and progesterone receptors in the hypothalamus and limbic system providing a morphological substrate for interaction between steroids and the opioid circuits in the brain. Both MOR and ORL-1 are receptors that respond to activation by endogenous ligands with internalization into early endosomes. This internalization is part of the mechanism of receptor desensitization or down regulation. Although receptor activation and internalization are separate events, internalization can be used as a temporal measure of circuit activation by endogenous ligands. This review focuses on the estrogen and progesterone regulation of MOR and ORL-1 circuits in the medial preoptic nucleus and ventromedial nucleus of the hypothalamus that are central to modulating sexual receptivity.

Index Entries: Sexual receptivity; μ-opioid receptor; orphanin FQ; nociceptin; OFQ; ORL-1; opioid receptor-like orphan receptor; lordosis; estrogen; progesterone; G protein-coupled receptors; β-endorphin; neuropeptide Y; NPY; endomorphin.

Introduction

Mu-opioid receptor (MOR) and opioid receptor-like receptor-1 (ORL-1) neuropeptide systems overlap with steroid responsive neural circuits. These circuits are part of a series of interconnected nuclei that are the central nervous system (CNS) substrates for integrative control of neuroendocrine regulation of reproduction and reproductive behavior in females. An important aspect of successful reproduction is coordination of behavioral and physiological events. The most

Received August 2, 2002; Accepted October 8, 2002
* Author to whom all correspondence and reprint requests should be addressed. E-mail: sinchak@mednet.ucla.edu

important of these are coupling of copulations with ovulation to maximize the possibility of fertilization. One of the challenges of studying steroid regulation of neural circuits controlling reproduction is determining when circuits are activated or inhibited.

MOR and ORL-1 belong to the superfamily of seven pass, transmembrane, G protein-coupled receptors (GPCR) *(1–8).* Ligand activation of GPCR is transduced through G proteins which regulate cellular activity by activating or inhibiting intracellular signaling cascades often through modulation of cAMP or Ca^{2+} levels. Another property of GPCR, including MOR and ORL-1, is that in response to agonist activation, these receptors are internalized into early endosomes and either recycled back to the plasma membrane or transported to lysosomes to be degraded. Although receptor activation and internalization are separate events *(9,10),* it is becoming increasingly clear that receptor internalization is required for certain types of signaling *(11–14).* For example, the mutation of Lys^{44} to Ala in dynamin, a protein involved in endocytosis, disrupts both the agonist-induced internalization of the δ-opioid receptor and stimulation of the extracellular signal-regulated protein kinase (ERK) signal transduction pathway *(14).* In either case, internalization can be used as a temporal and spatial measure of circuit activation by endogenous ligands *(15–17).* Antibodies against GPCR have been used to study neural circuit activation in a number of different GPCR and neural systems *(18–20).* This review primarily focuses on the estrogen and progesterone regulation of MOR and ORL-1 circuits in the medial preoptic nucleus (MPN) and ventral medial nucleus of the hypothalamus (VMH), respectively, which are important for regulating sexual receptivity.

Few neuropeptides in the CNS are as involved in so many aspects of regulatory reproduction as are the endogenous opioid peptides. As part of the hypothalamo-pituitary-gonadal axis, gonadal hormones and endogenous opioid peptides circuits interact to regulate the release of luteinizing hormone releasing hormone (LHRH) modulating luteinizing hormone (LH) release and ovulation; and as part of the lordosis regulating neural circuitry, they regulate reproductive behavior. Our hypothesis is that the gonadal steroids, estrogen and progesterone, orchestrate a temporal pattern of endogenous opioid peptide expression and release that activate cognate receptors within the limbic–hypothalamic circuit preventing, then facilitating, lordosis behavior.

Estrogen is known to act via several mechanisms to modulate circuits in the CNS:

1) Estrogen may act directly within a neuron to regulate the transcriptional activity of genes via a classic steroid receptor mechanism in which estrogen enters a target cell and binds with high affinity to estrogen receptor-α or -β. This steroid-receptor complex dimerizes and binds to estrogen-responsive elements, and in association with other transcription elements modulates the transcriptional activity of a specific gene (*21;* for review *see* ref. *22*). This type of regulation has been proposed for reproductively relevant neuropeptides such as enkephalin (ENK) and substance P *(23–25).*
2) Estrogen receptors may stabilize the fos-jun dimer at the AP-1 site *(26).*
3) Estrogen may act indirectly through transsynaptic mechanisms where steroids alter synaptic communication in one cell to regulate gene expression in a second cell through activation of signal transduction cascades as has been suggested for LHRH and cholecystokinin (*27–29;* but *see* refs. *30,31*).
4) Estrogen may not increase transcriptional activity but may stabilize mRNA levels within a cell *(32).*
5) Estrogen may also bind to and activate a putative receptor associated with the cell membrane which mediates signal-transduction pathways *(33–35).* Data suggesting cross-talk between these cell surface receptor-mediated signal-transduction pathways and classic steroid-induced genomic responses are just beginning to emerge (*33,36;* reviewed in ref. *37*). These plasma membrane-associated receptors can either modulate gene transcription through second messenger systems such as cAMP, or regulate nongenomic actions of estrogen which are too rapid to be compatible with the involvement of changes in transcription and translation (*34,38,39*; reviewed in ref. *40,41*).

6) Estrogen has been shown to bind to voltage-independent, poorly-selective cation channels that mediate the movement of cations across the membrane and therefore regulate changes in plasma-membrane polarity and the activation of calcium-dependent second messenger systems *(38).* Nongenomic, rapid effects of estrogen (in vivo and in vitro) have been suggested to account for the release of endogenous opioid peptide within the hypothalamus *(16,17).* The cellular actions of estrogen have been consolidated and described as a trinity of estrogen effects: membrane, cytosolic, and nuclear *(42).*

Steroid Regulation of Lordosis

Sexual receptivity is tightly regulated by the sequential release of estrogen and progesterone from the ovary. In ovariectomized rats, sexual receptivity can be induced by either estrogen alone or the sequential treatment with estrogen and progesterone. Although both steroid treatments induce sexual receptivity, several characteristics of the resulting behavior suggest that the mechanisms are different.

1) The dose of estrogen-only needed to induce sexual receptivity is higher than that needed when estrogen is supplemented with progesterone (reviewed in ref. *43*).
2) The repetitive estrogen treatment needed for maximal sexual receptivity results in a ramping of increasing sexual receptivity as measured by lordosis quotient (the number of lordosis divided by the number of mounts by the male multiplied by 100), whereas, estrogen + progesterone treatment produces a constant level of lordosis *(44).*
3) Progesterone receptors are not required for estrogen-only facilitation of lordosis *(45)* as evidenced by the fact that progesterone-receptor antagonists do not disrupt estrogen-only induced sexual receptivity (*46;* Fig. 1). 4) Estrogen-only induced lordosis has a later onset and longer window of sexual receptivity compared with estrogen + progesterone (*47;* reviewed in ref. *43*).

Following estrogen treatment, sexual receptivity cannot be facilitated for a period of almost 24 h. Progesterone starts to facilitate lordosis approx 20 h after estrogen treatment, and this estrogen + progesterone-induced sexual receptivity becomes maximal approx 24 h after estrogen *(17).* In estrogen-only animals, a large dose of estrogen in a single bolus injection is used to facilitate sexual receptivity; however, the onset is delayed approx 48 h after initial treatment, and the dose of estrogen required is higher than when receptivity is facilitated with subsequent progesterone *(48).*

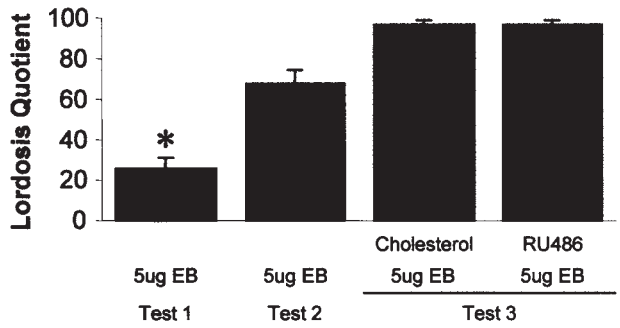

Fig. 1. Estrogen-induced sexual receptivity is not blocked by the progesterone receptor blocker RU486. Ovariectomized rats were treated with 17β-estradiol benzoate (5 μg) every 4 d and tested for sexual receptivity 48 h after each estradiol treatment (Test 1 and 2). This estrogen-priming regimen facilitates maximal sexual receptivity by the third cycle *(44).* Starting the morning of the third treatment of estradiol, animals were treated with either 1 mg cholesterol or 1 mg of RU486 for 3 d and tested for sexual receptivity (Test 3). * = Test 1 less than all other tests $p < 0.05$, Dunn's test (Kruskal-Wallis one way ANOVA on ranks H = 40.247, df = 3, $p < 0.001$).

MOR Regulation of Lordosis

MOR activation in the MPN inhibits lordosis *(17,49,50).* Other brain regions are implicated in MOR regulation of sexual receptivity, however, this review will focus on the MPN *(51–53).* Site-specific injection of MOR agonists, for example β-endorphin (β-END), endomorphin-1 (ENDO-1), or H-Tyr-D-Ala-Gly-N-Met-Phe-glycinol-enkephalin (DAMGO) into the MPN inhibits lordosis in female rats that have been made sexually receptive with estrogen + progesterone treat-

ment *(17,49,54).* Interestingly, antagonizing MOR activity in the MPN concurrently and immediately following estrogen treatment attenuates the subsequent progesterone facilitation of lordosis 48 h later, suggesting that an initial activation of MOR circuits inhibitory to sexual receptivity is an important process for the expression of maximal sexual receptivity *(55–57).*

ORL-1 Regulation of Lordosis

In contrast to the MPN, there is little or no MOR in the VMH *(58,59).* The VMH is one of the main nuclei driving sexual receptivity in the female rat *(60).* Recently, in attempting to find other opioid receptors, another GPCR that is closely related to the opioid receptors was discovered—the ORL-1 *(6–8,61).* This receptor system has high structural homology to the classical opioid receptors, especially the κ-opioid receptor (reviewed in refs. *62*). Its endogenous ligand is an opioid-like peptide, orphanin FQ (OFQ; also known as nociceptin; *63,64*). In the VMH, OFQ regulates lordosis. The facilitation of lordosis by site-specific injection of OFQ into the VMH is dose dependent, rapid, and long lasting; observed both 10 and 60 min after injection *(65).* Further, OFQ facilitation of lordosis is insensitive to VMH infusion of naloxone, a classical opioid-receptor antagonist *(65).* Since naloxone did not alter OFQ induced lordosis, the OFQ facilitation of lordosis in the VMH is not mediated through the downstream activation of MOR or other classical opioid receptors in the VMH. Although ORL-1 is not antagonized by naloxone, a *sine qua non* of classical opioid receptors, the ORL-1, will be considered a member of an expanded opioid-receptor family on the basis of structural similarity and cellular action *(66).* At this time, the only brain region that has been studied for OFQ regulation of reproduction is the VMH, although other lordosis-regulating brain regions have receptors for OFQ (reviewed below), and thus, the potential to regulate sexual behavior *(67).*

Estrogen Modulation of MOR Active and ORL-1 Active Peptides

There are at least four endogenous peptides that have affinity for and activate MOR: β-END, methionine-ENK (met-ENK), ENDO-1 and endomorphin-2 (ENDO-2). Both β-END and met-ENK are promiscuous, a result of the classical opioid peptides amino acid terminal (YGGFM/L) which allows for binding to both MOR and δ-opioid receptors. Although β-END binds to MOR and δ-opioid receptors with similar affinity, it has been considered the primary endogenous ligand for MOR *(68–71).* In a similar manner, met-ENK is considered the endogenous ligand for δ-opioid receptors, but also binds and activates MOR. The endomorphins have been proposed as the selective endogenous MOR ligands *(72,73).*

β-Endorphin

β-END is one of the cleavage products of proopiomelanocortin (POMC). The origin of β-END in the hypothalamus and limbic system is from cell bodies in the arcuate nucleus of the hypothalamus *(74).* β-END immunoreactive fibers are distributed throughout the hypothalamus, preoptic area (including the MPN), diagonal band of Broca, medial amygdala posterodorsal, (MeApd), bed nucleus of the stria terminalis (BST), which are areas involved in the regulation of reproduction and maternal behavior (reviewed in ref. *75*). The β-END circuit is extremely sensitive to estrogen control. Estrogen appears to have a biphasic effect on POMC expression, the steroid regulation of POMC expression is generally negative, but there is a complicated pattern. Estrogen transiently induces expression *(76)* and then expression is attenuated *(77).* Progesterone treatment on the second day after estrogen further suppresses POMC expression coinciding with the period of sexual receptivity and the initiation of the LH surge. At the peak of the LH surge, estrogen and progesterone augment POMC expression *(78,79).*

In terms of reproductive behavior, the β-END story is also complex. Most of the POMC mRNA expressing neurons that project to the medial portion of the MPN are located in the rostral portion of the arcuate nucleus *(80).* In the medial preoptic area, β-END immunoreactive fibers are distributed in a medial to lateral gradient. The periventricular preoptic nucleus along the third ventricle has the greatest density of immunoreactivity and the density decreases laterally such that the lateral preoptic area has very few fibers (reviewed in ref. *75*). Estrogen treatment of ovariectomized rats does not alter the relative density of β-END immunoreactivity, however, a 27 h exposure to estrogen + progesterone increases the density of β-END immunoreactive fibers. In cycling females, β-END immunoreactivity increases on the day of estrus and is lower on all the other days of the cycle *(81).* One mechanism that would account for these results is that estrogen induces β-END release which decreases tissue levels and immunoreactivity in the MPN. Subsequent treatment with progesterone inhibits further release and stimulates the expression of the POMC mRNA *(78).* This interpretation of the results is consistent with receptor internalization that is dependent on release of endogenous MOR-active opioid peptides such as β-END in the MPN *(16,17,82).*

Interestingly, the expression of β-END mRNA is regulated by estrogen, but only 4–10% of β-END neurons in the arcuate nucleus express estrogen receptor-α *(83–85).* A similar low number of β-END neurons have estrogen receptor-β *(86,87).* Thus, estrogen may be regulating POMC expression via genomic and/or nongenomic mechanisms. Moreover, like the initial upregulation of preproenkephalin *(88),* the initial expression of POMC mRNA expression may be due to stress *(89–91).* In both cases, the initial rise of expression is not blocked by the selective estrogen-receptor antagonist tamoxifen *(76,89).* In the case of preproenkephalin, its initial expression is eliminated by adrenalectomy *(89).*

Endomorphins

The recently characterized endomorphins, ENDO-1 and ENDO-2, have high affinity and selectivity for MOR *(72).* The hypothalamus contains some of the highest concentrations of these MOR-selective endogenous opioid peptides in the CNS *(72).* ENDO-1 and -2 immunoreactive fibers are distributed throughout the nuclei of the lordosis regulating limbic–hypothalamic circuit *(92–94).* Although distribution studies have not been done in female rats, in the male rat, the distribution of endomorphins suggests ENDO-1 may be more involved in reproduction than ENDO-2. ENDO-1 immunoreactive fibers are distributed in the MPN and periaqueductal gray with sparse staining in the MeApd *(92).* ENDO-2 fibers are sparsely distributed in the MPN but absent in the MeApd. ENDO-1 and ENDO-2 immunoreactive cell bodies are found in the dorsal region of the arcuate nucleus, dorsomedial nucleus of the hypothalamus, and in a cluster of cells capping the medial dorsal VMH ventral to the dorsomedial nucleus of the hypothalamus *(92–95).* Very little is known about the regulation of the endomorphins, since the gene or gene product from which these peptides originate is not known. Since the distributions of ENDO-1 and ENDO-2 immunoreactive perikarya and estrogen or progesterone receptor positive neurons overlap minimally, it is unlikely that endomorphins are regulated directly via classical estrogen- and progesterone-receptor mechanisms *(87,96).* However, the localization of ENDO-1 immunoreactive fibers in the MPN and MeApd suggests that ENDO-1 may be regulated by these steroids through indirect or nonclassical mechanisms. Whether this indirect estrogen response is through estrogen receptor-α or -β or via a different mechanism (discussed below) remains to be evaluated.

Enkephalins *(ENK)*

ENK have been proposed as positive modulators of lordosis (50). Preproenkephalin mRNA

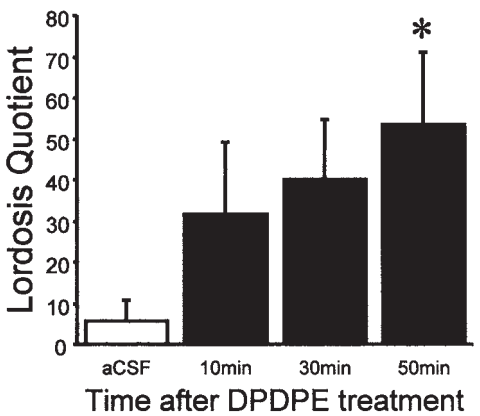

Fig. 2. Activation of DOR in the VMH-facilitated lordosis in ovariectomized female rats treated with 17β-estradiol benzoate (2 μg) every 4 d for three "cycles." In response to a stimulus male, these rats had very low levels of lordosis after microinjection of artificial cerebrospinal fluid (aCSF) into the VMH (vol: 0.5 μL). Microinjections of 5 n*M* DPDPE, a DOR agonist, into the VMH-increased sexual receptivity. Significant facilitation of lordosis was measured at the 50 min time point suggesting that there is a delay in the DPDPE facilitation of lordosis. Results are means ± SEM of 6 trials, one-way repeated measures ANOVA df = 5.23, F = 3.255; $p = 0.05$. * Indicates significantly greater than aCSF at the $p < 0.05$ level (Student-Newman-Keuls).

message levels significantly increase 48 h after estrogen treatment in the VMH *(25,97),* MPN *(98),* and MeApd *(89,99).* In the VMH, a significant proportion of ENK immunoreactive cells are estrogen receptive *(100)* indicating a direct activation of preproenkephalin transcription through estrogen-activated estrogen receptors. Although it has been suggested as a modulator of lordosis, the time course of preproenkephalin mRNA expression in the VMH and MPN may be too slow to affect behavior. The peak level of message expression and lordosis are well correlated, but in order to be active, the preproenkephalin mRNA must be translated into the biologically active ENK peptides and released. It should be noted, however, that specific activation of δ-opioid receptor in VMH does result in increased lordosis (Fig. 2).

Orphanin FQ/Nociceptin

A cDNA-encoding OFQ has been characterized and encodes a hectadecapeptide similar in structure to dynorphin A *(63,64,101),* but OFQ lacks the NH_2 terminal tyrosine necessary for activation of the MOR, κ-opioid receptor, or δ-opioid receptor *(63,64,101).* OFQ specifically binds to ORL-1 with much higher affinity than the other endogenous opioid peptides or opioid agonists *(102–107). In situ* hybridization, immunohistochemical and OFQ-binding studies demonstrate that the distributions of OFQ and its receptor ORL-1 overlap with steroid-responsive regions throughout the hypothalamus and limbic system that regulate reproduction *(8,61,65,67,108–112).* These regions include the medial amygdala, BST, MPN, and arcuate nucleus. Despite this distribution, there are a paucity of studies examining the steroid regulation of the OFQ/ORL-1 circuits and their role in reproduction. OFQ mRNA expression is conspicuously absent in the VMH but does facilitate lordosis when injected into the VMH which expresses ORL-1 mRNA as discussed below *(65).* In brain slices containing the mediobasal hypothalamus, bath application of OFQ hyperpolarizes 81% of the neurons recorded from in the VMH *(113).* Additionally, 75% of these neurons were also hyperpolarized by met-ENK *(113).* Consistent with the notion that ORL-1 is insensitive to naloxone, naloxone blocked only the hyperpolarization induced by met-ENK, but not by OFQ. Our studies also demonstrated that naloxone does not affect OFQ facilitation of lordosis in the VMH. Together these intriguing data imply that activation of either ORL-1 or δ-opioid receptors in the VMH facilitates lordosis, suggesting that these systems may converge on specific VMH neurons to regulate sexual receptivity. Preliminary studies indicate that estrogen positively modulates OFQ and ORL-1 expression in limbic–hypothalamic circuits that are important for regulating lordosis *(65,67).*

MOR in the Hypothalamus

Distribution of MOR

Despite several studies, the sex-steroid regulation of opioid receptors and their function is murky. For example, no consensus has emerged about how sex steroids regulate MOR binding. In the medial preoptic area, sex steroids have been reported to decrease, increase, or not change opioid-receptor binding *(114–119).* Two possible reasons for these differing results are the use of different opioid ligands and different steroid treatments (both duration and amount). Apparently low levels of estrogen increase MOR binding, and high levels decrease the number of MOR *(120).* In homogenates of rat hypothalamus, maximal binding and affinity of MOR fluctuate and are differentially regulated across the estrous cycle *(121).* MOR B_{max} is elevated on diestrus d 2 and peaks on the morning of proestrus and then dramatically decreases during the afternoon and evening of proestrus. Following the LH surge on estrus, MOR binding appears to vacillate between moderate to low levels. On diestrus d 1 the number of MOR is lowest. Interestingly, on proestrus when binding is low, MOR affinity is also decreased, whereas on diestrus d 1 when binding is lowest, MOR affinity is the highest *(121).* However, estrogen replacement (50 μg or 2 μg) given to ovariectomized rats results in no change in MOR binding in MPN for 30 h after treatment *(16,122).*

Activation of MOR

Previous studies have concentrated on steroid modulation of opioid-receptor ligand mRNAs, MOR message and number of binding sites. However, these methods do not reveal when the circuit is activated. MOR internalization following agonist activation, and the subcellular distribution of the receptor can be used as a marker of receptor activation in response to a stimulus *(16,123–132).*

There are two distinguishable processes of ligand-initiated GPCR regulation that do not involve expression: desensitization and downregulation *(133–137).* For the opioid receptors, both processes are stimulated by peptide and certain alkaloid agonists but are distinguishable in terms of their time course and change in receptor number *(132,138–140).* Desensitization does not alter receptor number, whereas downregulation reduces receptor number. Desensitization is associated with the rapid internalization (translocation) of receptors following agonist binding while downregulation is a slower process *(132,138–140).* Upon agonist binding, the agonist-receptor complex is phosphorylated and rapidly internalized into early endosomes. In this intracellular compartment, the receptor is dissociated from its agonist and either returned to the plasma membrane or degraded (Fig. 3). The internalization and recycling to the membrane occurs without loss of receptor number. Thus, internalization of GPCR, visualized by immunocytochemistry, can be

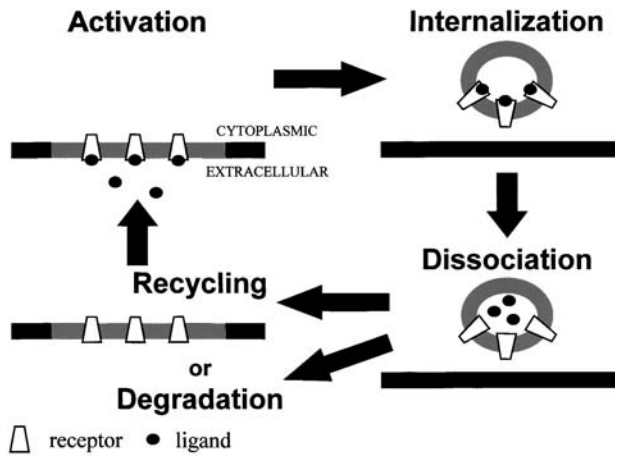

Fig. 3. Schematic of agonist-induced internalization of G protein-coupled receptors. Unbound receptors are present on the plasma membrane. Agonist binding to the membrane-bound receptor induces a series of events culminating in the sequestration of the receptor in early endosomes. In these highly acidic compartments, the ligand is dissociated from the receptor. The receptor is either recycled to the membrane, ready to be activated again or degraded. The degradation of receptors is referred to as downregulation of the receptor.

used as a marker of receptor activation *(123,128–132,141).* Once internalized, the receptor can also be degraded. This receptor downmodulation is relatively slow and is associated with a loss of binding sites *(133,134,136,137,142).*

Antibodies directed against a synthetic peptide (LENLEAETAPLP) corresponding to amino acids 387–398 of the intracellular carboxyl terminal of MOR *(108,143)* revealed MOR immunoreactive fibers throughout the medial MPN, BSTp, MeApd, the paraventricular nucleus, dorsomedial nucleus, periventricular zone, and median eminence *(16,82,141).* Similarly, antibodies against MOR epitopes have been used to map MOR subcellular distribution *(143–145).* When MOR circuits are less active or inhibited by antagonists, MOR immunostaining is generally located on the plasma membrane of cell bodies and dendrites *(16,82,131,144,146–149).* MOR immunoreactivity is internalized when activated by β-END and endomorphins *(10,17,139,150,151).*

Although receptor internalization is a marker for ligand activation of GPCR, the ability of a ligand to induce GPCR internalization does not necessarily correlate with the ability of a ligand to stimulate receptor-mediated second-messenger signaling, G protein-coupling efficiency, or activation *(10,139).* For example, a comparison of the ability of three alkaloid agonists to internalize MOR showed that etorphine induces maximal MOR internalization, fentanyl induces 66% and morphine only 17% of maximal MOR internalization *(139).* Yet, morphine and fentanyl have equivalent potency and efficiency in producing maximal inhibition of adenylate cyclase, and activation of G protein-binding *(10).* For endogenous opioid peptides, the ability to internalize MOR are well correlated with activation of MOR *(15,17,139,150,152).*

Sex Steroid Regulation of MOR Activation/Internalization

Agonist-induced GPCR internalization was used to investigate spatial and temporal parameters of ovarian-steroid activation of MOR circuits important for sexual receptivity in the female rat *(16,17,82).* Estrogen, like MOR ligands (e.g., etorphine or ENDO-1), rapidly induces internalization (activation) of MOR , however, the estrogen-induced time course of MOR internalization is more prolonged *(16,17).* Estrogen-induced MOR internalization is restricted to estrogen-responsive cell groups suggesting that estrogen receptors may mediate this internalization. Translocation of MOR immunoreactivity is first observed in the MPN and MeApd 15 min after estrogen treatment *(16).* The pattern of MOR internalization lasted for at least 30 h *(17).* Confocal microscopy confirmed that estrogen-induced MOR translocation from the plasma membrane to intracellular sites thought to be early endosomes (Fig. 4). The internalization of MOR immunoreactivity and concentration of the immunoreactivity in neural processes causes the reduction in diameter due to the massive internalization of membrane *(130)* making them more visible and showing up as an increased number of filled processes after etorphine or estrogen treatment, which was quantified as an increase in MOR immunoreactive fiber density (Fig. 5; *16*). The microscopic level, at the light the distinct and diffuse immunocytochemical staining patterns of another GPCR, and the somatostatin sst2A receptor were correlated at ultrastructural level with the receptors being either internalized (distinct) or associated with the membrane respectively (diffuse; *153*).

Statistically, estrogen treatment increased MOR-fiber density within 30 min (Fig. 5). The increase in MOR-fiber density/internalization peaked at 4 h and was maintained for at least 30 h *(16,17).* MOR-fiber density returned to basal levels indicating that MOR was no longer internalized at the 48 h postestrogen treatment time point (Fig. 5). In estrogen-only treated rats, lordosis is not exhibited until approx 48 h after treatment, a time point at which the MOR-mediated inhibition has been relieved. Progesterone treatment of estrogen primed rats accelerated the onset of lordosis, facilitating lordosis 30 h after estrogen treatment *(17).* Sim-

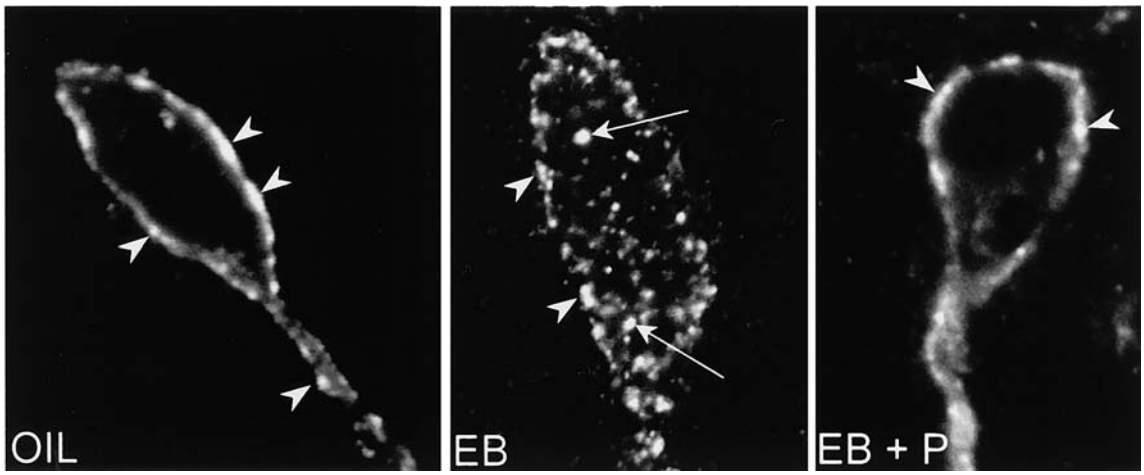

Fig. 4. Confocal projections of MOR immunoreactive processes in the medial part of the medial preoptic nucleus from ovariectomized rats treated with either oil (OIL), 17β-estradiol benzoate (EB) + oil or EB + progesterone (EB + P) and killed 30 h after initial oil or EB treatment. The second oil or progesterone treatment was given 26 h after initial treatment. MOR immunoreactivity was associated with the plasma membrane in the oil treated rats (arrow heads). In the EB-treated, the MOR immunoreactivity was translocated into early endosomes in the interior of the cell (arrows) indicating that MOR activation by the endogenous release of MOR ligand. MOR immunoreactivity returned to the plasma membrane (arrow heads) in EB + P treated rats indicating that subsequent progesterone treatment that facilitates sexual receptivity inhibits the release of MOR ligand and MOR internalization/activation (from refs. *16,17*).

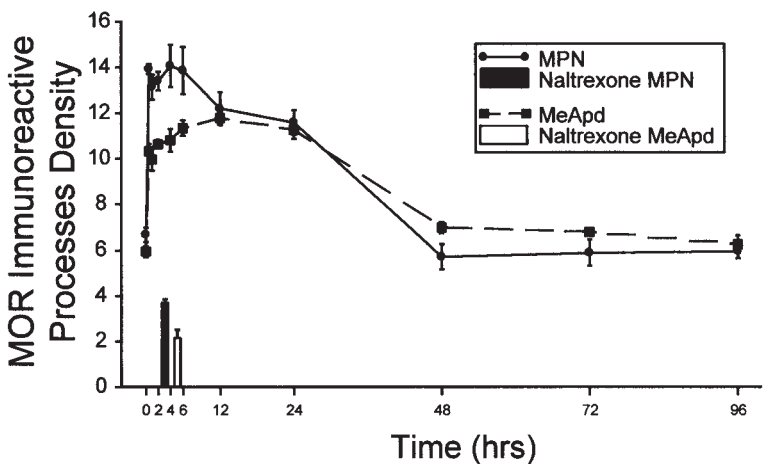

Fig. 5. Graphic representation of the density of MOR immunoreactive processes in the medial part of the medial preoptic nucleus (MPN, solid line) and the posterodorsal medial amygdaloid nucleus (MeApd, dashed line) following 17β-estradiol benzoate (EB; 50 μg) treatment. The moment of EB injections is time 0. The density of distinct MOR immunoreactive processes is significantly elevated by 30 min and peaks at 4–6 h. The density of MOR immunoreactive processes is basal by 48 h. The bars at the 4 h time point illustrate the density of MOR immunoreactive processes in the MPN (solid bar) and MeApd (open bar) in EB treated ovariectomized rats pretreated with naltrexone 24 and 4 h prior to perfusion. The density of MOR immunoreactive processes in the naltrexone treated rats is significantly lower than in ovariectomized rats in the MPN and MeApd indicating a basal level of MOR activation is normally present. Each point is the mean + SEM (n = 4/time point; ref. *16*).

ilarly, subsequent progesterone treatment, 26 h after estrogen reversed the estrogen-induced MOR internalization. This blockade of internalization is correlated with a progesterone facilitation of lordosis *(17)*. These data suggest that progesterone antagonizes the action(s) of estrogen that activate MOR in the MPN.

Does estrogen act on MOR to induce internalization? Based on binding data *(122)*, naloxone blockade *(16)*, and the effects of estrogen and progesterone *(17)*, we hypothesize that estrogen induces a rapid and prolonged release of endogenous MOR ligand(s), and subsequent progesterone stimulation inhibits the release of endogenous MOR ligand(s), relieving MOR-inhibitory tone, facilitating lordosis. This idea is further supported by the congruence of MOR activation by estrogen, with the period of absolute refractoriness of sexual receptivity and progesterone-induced receptivity (MOR inactivation). Further, the reduction in MOR activation by 48 h of a high dose of estrogen correlates with the onset of sexual receptivity produced by estrogen-only treatment, suggesting that relief of MOR inhibition in the MPN is an important step in achieving sexual receptivity. Although there may be alternative explanations for the estrogen-induced translocation of MOR, the most plausible is that estrogen and progesterone regulate the release of endogenous peptide, since naloxone prevents estrogen-induced internalization *(16)*.

To determine if activation of MOR in the MPN is correlated with inhibition of lordosis by opioids, estrogen and progesterone primed rats were infused intracerebroventricularly with either the MOR agonist, DAMGO, or ENDO-1. Both MOR-specific opioids inhibited lordosis within 10 min and induced MOR internalization with a similar time course to estrogen. Additionally, infusion of DAMGO or ENDO-1 in the MPN of estrogen + progesterone primed rats inhibits lordosis, demonstrating that MOR are available and able to be activated by MOR ligands, indicating that progesterone blocks release of endogenous MOR ligand(s) into the MPN *(17)*. During the period of estrogen-induced MOR internalization, MOR binding is not affected as measured by an autoradiographic-binding assay that measures the total number of receptors in tissue *(16,122)*. However, after prolonged estrogen exposure (≥ 48 h), the number of opioid-binding sites in the MPN is reduced *(115,117,154,155)*. Thus, the decrease in MOR-binding levels indicates that internalization is followed by downmodulation *(128,156)* and may be part of the mechanism involved in sexual receptivity induced by estrogen-only treatment.

MOR Internalization During the Estrous Cycle

An important question is whether this type of internalization takes place under physiological conditions that are associated with reproduction. In the cycling female rat, levels of MOR internalization in the MPN fluctuate throughout the estrous cycle (Fig. 6). MOR internalization/activation is coincident with periods of lower sexual receptivity. As estrogen levels rise, MOR are internalized on diestrus d 1 and 2, when the female is not receptive. Potentially, this MOR activation prior to ovulation may be required for complete sexual receptivity *(56,57)* and maximal fertility. The lowest levels of internalization (lowest MOR activation) are on the afternoon and evening of proestrus, when rats are sexually receptive. Finally, MOR internalization is increased again on estrus suggesting that MOR activation may be important for terminating sexual receptivity.

Site of Estrogen Action to Stimulate Release of MOR Endogenous Ligand(s)

Are estrogen actions on MOR directly on neurons that release β-END or endomorphins? In terms of the endomorphins, as reviewed above, distributions of endomorphin immunoreactive-cell bodies and estrogen-receptor neurons overlap minimally *(92,94,157,158)*. Currently, no data exist concerning the colocalization of estrogen receptors and endomorphins nor the distribution of

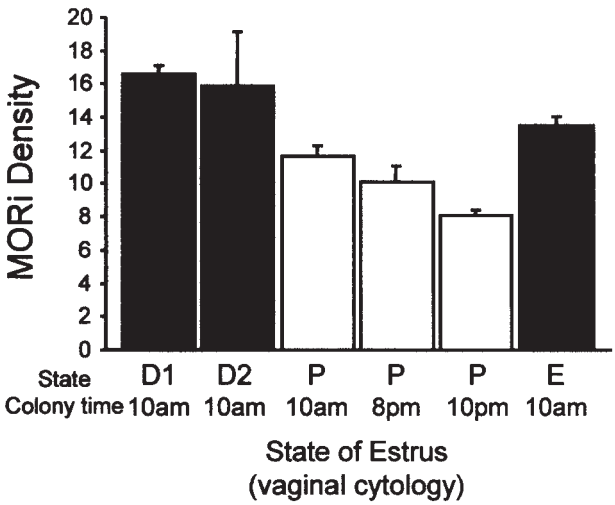

Fig. 6. Histogram of MOR immunoreactive fiber density in the medial part of the MPN during the estrous cycle. In this study (n = 3), animals were killed at 10:00 am on each day of the estrous cycle (D1 = diestrus, d 1; D2 = diestrus, d 2; P = proestrus; E = estrus) and at 8:00 pm and 10:00 pm on proestrus. MOR immunoreactive fiber density is a measure of MOR internalization and MOR activation. During times when sexual receptivity is low (D1, D2, and E), the density of MOR immunoreactive processes is high. On proestrus there was a decrease in MOR immunoreactive processes density indicating fewer internalized MOR. This decrease coincides with elevated lordosis. Thus, MOR activation is inversely correlated with lordosis in a cycling rat (*200*).

endomorphins in the female rat to determine whether estrogen directly acts on the endomorphins. A relatively low level of estrogen receptor and β-END colocalization suggests that estrogen acts through an interneuron to activate MOR in the MPN. A possible candidate for the interneurons that estrogen regulates, are the neuropeptide Y (NPY)-expressing cells in the arcuate nucleus. According to this hypothesis, estrogen would release NPY in the arcuate nucleus to activate β-END neurons that project to the MPN. The NPY-Y1 receptor is colocalized in a population of arcuate nucleus β-END neurons *(159,160).* Like MOR-active endogenous opioid peptides, activation of NPY receptors inhibits lordosis *(159).* Infusion of NPY into the third ventricle of ovariectomized rats, induces internalization of NPY-Y1 receptor in the arcuate nucleus and inhibits lordosis *(160).* Like MOR in the MPN, estrogen rapidly induces internalization of the NPY-Y1 receptors in the arcuate nucleus, and subsequent progesterone treatment reverses the NPY-Y1 estrogen-induced internalization. These results indicate that one pathway through which estrogen activates MOR in the MPN to regulate lordosis is through stimulating release of NPY in the arcuate nucleus which activates NPY-Y1 on β-END neurons that project to and release β-END in the MPN.

Estrogen Receptor-α is Necessary for the Estrogen-Induced Internalization of MOR

Estrogen induces both rapid and prolonged activation of MOR. Tamoxifen decreases estrogen-induced MOR internalization as measured by MOR immunoreactive fiber density (12.9 ± 0.3 immunoreactive fibers per 100 μm) compared estrogen-treated animals (16.7 ± 1.3 immunoreactive fibers per 100 μm). Classically, tamoxifen blockade of estrogen actions suggests activation of an intracellular receptor (*26,161;* but *see* refs. *39,162).* Which estrogen receptor is activated and whether estrogen is acting via nongenomic and/or genomic mechanism(s) to regulate MOR activation are intriguing questions *(163–170).* Estrogen appears to be acting via estrogen receptor-α to induced MOR internalization, since in the estrogen receptor-α "knock out" mouse (ERαKO; *163,164*) estrogen does not induce MOR internalization in the MPN or MeApd, whereas MOR are internalized in both wildtype or estrogen receptor-β disrupted mice *(171).* However, intracerebroventricular infusion of ENDO-1 induced equivalent MOR internalization in ERαKO and wildtype mice. These results indicate that MOR expression does not require estrogen receptor-α during development and MOR are

functional. Moreover, these results suggest that estrogen receptor-β is not involved in the estrogen activation of the MOR-circuit in the MPN *(171).*

The prolonged MOR activation induced by estrogen acting via estrogen receptor-α is suggestive that estrogen is altering cellular activity through genomic mechanisms. However, the rapid estrogen-induced MOR internalization (within 15 min), suggests that the initial actions of estrogen action are via a rapid nongenomic mechanism(s) which may also be responsible for the prolonged effects of estrogen. Although estrogen receptor-α and -β are classically described as transcription factors, they also have the ability to be biologically active when associated with the plasma membrane. A recent study demonstrates that estrogen receptor-α may be targeted to the plasma membrane and activate adenylate cyclase and IP3-mediated intracellular signaling pathways *(34).* These authors reported that Chinese hamster ovary (CHO) cells, transfected with either estrogen receptor-α or estrogen receptor-β have both intracellular and membrane-associated estrogen receptors. In terms of estrogen-induced internalization of MOR, an estrogen receptor-α associated with the membrane that initiates intracellular cascades which release MOR endogenous opioid peptides would explain our results. An alternative explanation for the prolonged activation of MOR by estrogen is that estradiol benzoate remains in the animal for a prolonged period and continues to stimulate MOR-active peptide release, internalizing MOR.

Estrogen Alters GPCR G Protein Functional Coupling

Agonist binding of MOR activates pertussis toxin insensitive Gi/Go type G proteins *(172–174)* that stimulate intracellular-signaling cascades inhibiting adenylate cyclase *(175–177),* increasing potassium-channel conductance *(178,179)* and reducing calcium-channel conductance *(180–182).* Estrogen regulates signal transduction via G proteins either directly or indirectly (reviewed in refs. *183,184),* indicating that estrogen regulation of signal-transduction mechanisms is mediated by rapid nongenomic mechanisms, where estrogen receptors are located in the cytoplasm or associated with the plasma membrane (*33–35,185–188;* reviewed in refs. *41,42,183*).

The [^{35}S]GTPγS-binding assay has been used to identify receptor-activated G proteins by MOR *(189–194).* This assay is based on the fact that in the inactive state, the α subunit of the G protein has a relatively high affinity for GDP over GTP. Activation of receptors by an agonist shifts the α subunit to a higher affinity state for GTP compared with GDP. The assay uses excess GDP to shift the G proteins to receptor-uncoupled state and lower basal activity. Addition of [^{35}S]GTPγS and an agonist shifts the affinity of the G protein from GDP to GTP and the activated G protein binds the nonhydrolyzable [^{35}S]GTPγS.

We used this assay to demonstrate that estrogen can alter the functional coupling of MOR to its G protein. Ovariectomized rats were treated with estradiol benzoate (50 μg) or oil systemically for 4 h and then the medial preoptic area (containing the MPN) and the mediobasal hypothalamus (containing the arcuate nucleus and VMH) were processed for [^{35}S]GTPγS binding using 1 μ*M* morphine as a MOR agonist. The level of [^{35}S]GTPγS binding to mediobasal hypothalamic membranes was higher in oil and EB-treated rats compared with binding to the medial preoptic area membranes. Estrogen significantly increased morphine-induced [^{35}S]GTPγS binding in the medial preoptic area but not in the mediobasal hypothalamus (Fig. 10). This lack of estrogen effect on GTPγS binding in the mediobasal hypothalamus was previously reported *(195).* Our results suggest that estrogen alters the functional coupling of MOR in the medial preoptic area, but not in the mediobasal hypothalamus. These results indicate that estrogen may increase the efficacy of MOR such that when estrogen is elevated, MOR-active endogenous peptides are released and couple more efficiently to the inhibitory second-messenger cascades within MOR neurons to inhibit activity of MOR cells. In the

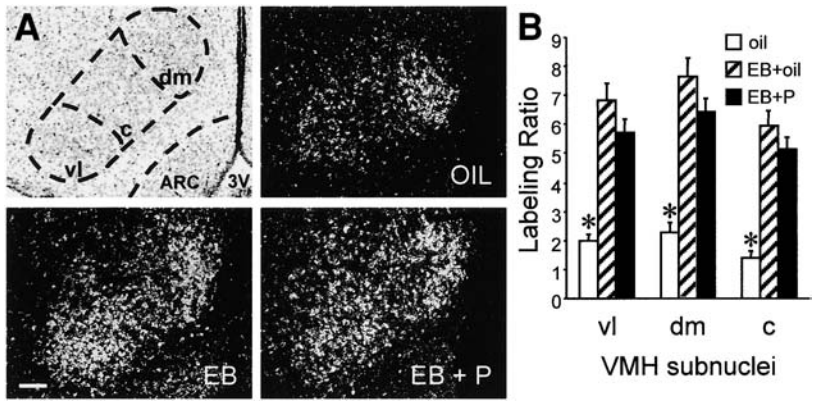

Fig. 7. **(A)** Darkfield photomicrographs of the effects of estrogen and progesterone on ORL-1 mRNA expression in the three subnuclei of the VMH (vl = ventrolateral; dm = dorsomedial; c = central) in ovariectomized rats treated with either 17β-estradiol benzoate (2 μg) and oil vehicle (EB + oil), 2 μg EB and 500 μg of progesterone (EB + P) or twice with oil vehicle (oil). The subsequent progesterone or oil treatments were given 26 h after the initial treatment, and tissue was collected 30 h after the initial EB or oil treatment and processed for *in situ* hybridization. Scale bar = 100 μm. **(B)** ORL-1 mRNA labeling ratio (mean +/– SEM) in the three subnuclei of the VMH. * = Significantly less than other treatments within subnuclei (Bonferroni *t*-test, $p < 0.001$).

absence of estrogen, both ligand activation of the receptor and its coupling to second messengers is attenuated. These data explain the estrogen-induced inhibition of medial preoptic area neurons *(196,197),* and places the MOR-mediated inhibition in a functional context for regulating reproduction.

Opioid Receptor-Like Receptor in the Hypothalamus

Distribution of Opioid Receptor-Like Receptor

ORL-1 immunoreactivity and mRNA expression are distributed throughout, but not limited to, the limbic–hypothalamic lordosis circuit *(61,108,112,198,199).* In the limbic hypothalamic system of the rat, ORL-1 mRNA expression and OFQ binding are present in the anteroventral periventricular nucleus, MPN, BST, paraventricular nucleus, suprachiasmatic nucleus, supraoptic nucleus, MeApd, and VMH *(6,7,61,67,110,112,199).* ORL-1 mRNA expression and immunoreactivity are located throughout the rostral-caudal extent of the VMH, however, ORL-1 mRNA and peptides are more concentrated in the dorsal medial part of the nucleus (Fig. 7). In the female rat, a 2 μg dose of estrogen that does not induce lordosis *(65)* increased both the expression of ORL-1 mRNA (Fig. 7; *67*) and area of ORL-1 immunoreactivity in the VMH 30h after treatment (Fig. 8; *65*). Subsequent progesterone treatment that facilitates lordosis affected neither ORL-1 expression nor the area of ORL-1 immunoreactivity in the VMH *(67,198),* suggesting that only estrogen regulates ORL-1 expression in the VMH.

Steroid Activation/Internalization of ORL-1 in the VMH

Using antibodies generated against the unique amino terminus of ORL-1 (MESLFPAPFWEVLYGSHF), the subcellular distribution of ORL-1 immunoreactivity in the VMH of ovariectomized rats treated with estrogen or estrogen + progesterone was examined *(65,198).* The majority of staining was restricted to cellular processes confirming earlier reports *(108,111).* A low dose of estrogen increases

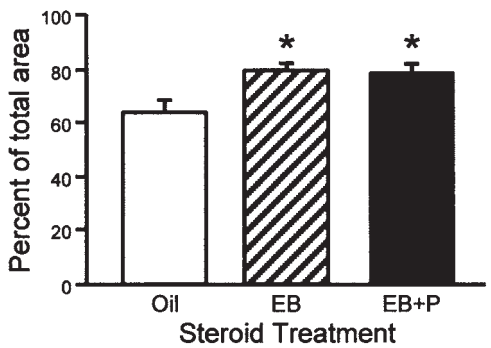

Fig. 8. Total area of distribution of ORL-1 immunoreactivity in the VMH of ovariectomized animals treated with oil, 17β-estradiol benzoate + oil (EB) (2 μg once every 4 d for 3 cycles) or EB + P (500 μg P 26 h after EB treatment). Animals were perfused 30 h after the last EB or oil injection. The VMH was sampled at three rostrocaudal sections at the level of the VMH ventrolateral, and the average area of specific ORL-1 staining was determined by computer-assisted morphometry (as described in refs. *65,201*). Data are mean ± SEM of four to six animals per group. * = $p < 0.05$ vs oil. No differences were noted between EB and EB + P groups (refs. *65,67*).

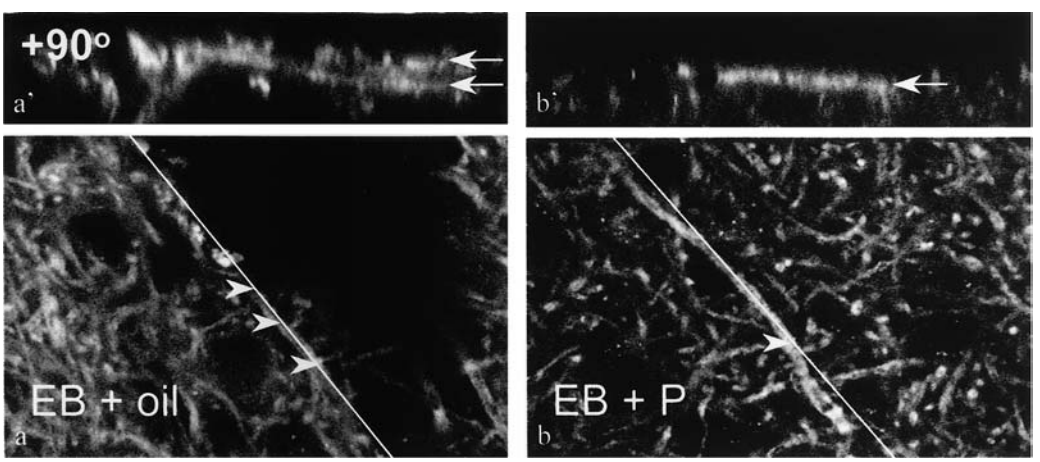

Fig. 9. Confocal projections of ORL-1 immunoreactive fibers in the VMH in ovariectomized animals treated with 17β-estradiol benzoate (2 μg) and 26 h later oil (EB + oil; a,a′), or 17β-estradiol benzoate (2 μg) and 26 h later progesterone (EB + P; **b,b′**). Brains were collected 30 h after the initial treatment. Bottom photomicrographs **(a,b)** illustrate the axis of optical Z-sectioning (line, arrowheads). The Z-section produced a cross-section of the fibers along the location of the lines in **a′** and **b′.** The image is rotated 90 degrees and projected in the top panel **(a′,b′).** In EB + oil-treated ovariectomized animals the OFQ immunoreactivity is associated with the membrane (arrows in **a′**). In EB + P treated animals the ORL-1 immunoreactivity is internalized (arrow in **b′;** *198*).

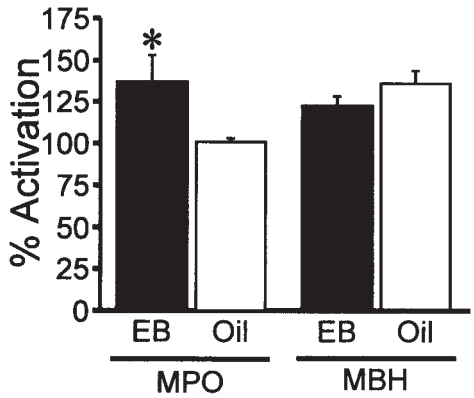

Fig. 10. Effect 17β-estradiol benzoate (EB) on morphine, a preferential MOR agonist, [^{35}S]GTPγS-binding in rat medial preoptic area (MPO) and mediobasal hypothalamus (MBH). EB increased G-protein functional coupling in the MPO, but not MBH. Membranes were incubated in 1 μ*M* morphine for 60 min at 30°C in the presence of 20 μ*M* GDP and 0.05 n*M* [^{35}S]GTPγS. Nonspecific binding was determined in the presence of 10 μ*M* unlabeled GTPγS. EB treatment of ovariectomized rats increased [^{35}S]GTPγS in the MPO which contains the MPN, but did not alter the binding in the MBH which contains the VMH. Results are means ± SEM of 6 animals. * = Greater than Oil treated within MPO region $p < 0.05$.

ORL-1 expression, but does not induce activation of ORL-1 as measured by internalization or sexual receptivity *(65,67,198).* As described previously, progesterone treatment that induces sexual receptivity does not increase the area of ORL-1 immunoreactivity but does induce internalization of ORL-1 immunoreactivity (Fig. 9). Progesterone treatment and microinjection of OFQ produced similar facilitation of lordosis in estrogen-primed rats, suggesting that endogenous OFQ may be released by progesterone in the VMH to facilitate lordosis. Thus, it appears that estrogen upregulates ORL-1 expression, but does not induce release of OFQ, whereas subsequent progesterone induces the release of OFQ activating and internalizing ORL-1 leading to a facilitation of lordosis *(198).*

Summary

Endogenous opioid peptides positively and negatively modulate sexual receptivity. These effects are receptor-specific and site-specific: MOR inhibit lordosis in the MPN and δ-opioid receptors and ORL-1 facilitate lordosis in the VMH. The activation of specific inhibitory (MOR) circuits and facilitatory circuits (δ-opioid receptors and ORL-1) circuits is determined by the sex steroid hormone milieu. Estrogen activates the MOR by increasing the release of endogenous opioid peptides such as β-END and ENDO-1, and increasing the postsynaptic coupling of the MOR to G proteins. In the VMH, estrogen increases the expression of ORL-1. When progesterone stimulates the CNS, it turns off the MOR circuit (MOR inactivation) and releases OFQ, facilitating lordosis (Fig. 11). Estrogen and subsequent progesterone treatment regulate a balance of MPN vs VMH activation that determines whether the animal is sexually receptive.

Acknowledgments

Drs. Paul Popper and Richard Mills for their stimulating discussions and insightful comments on the manuscript. Dr. Clair Eckersell,

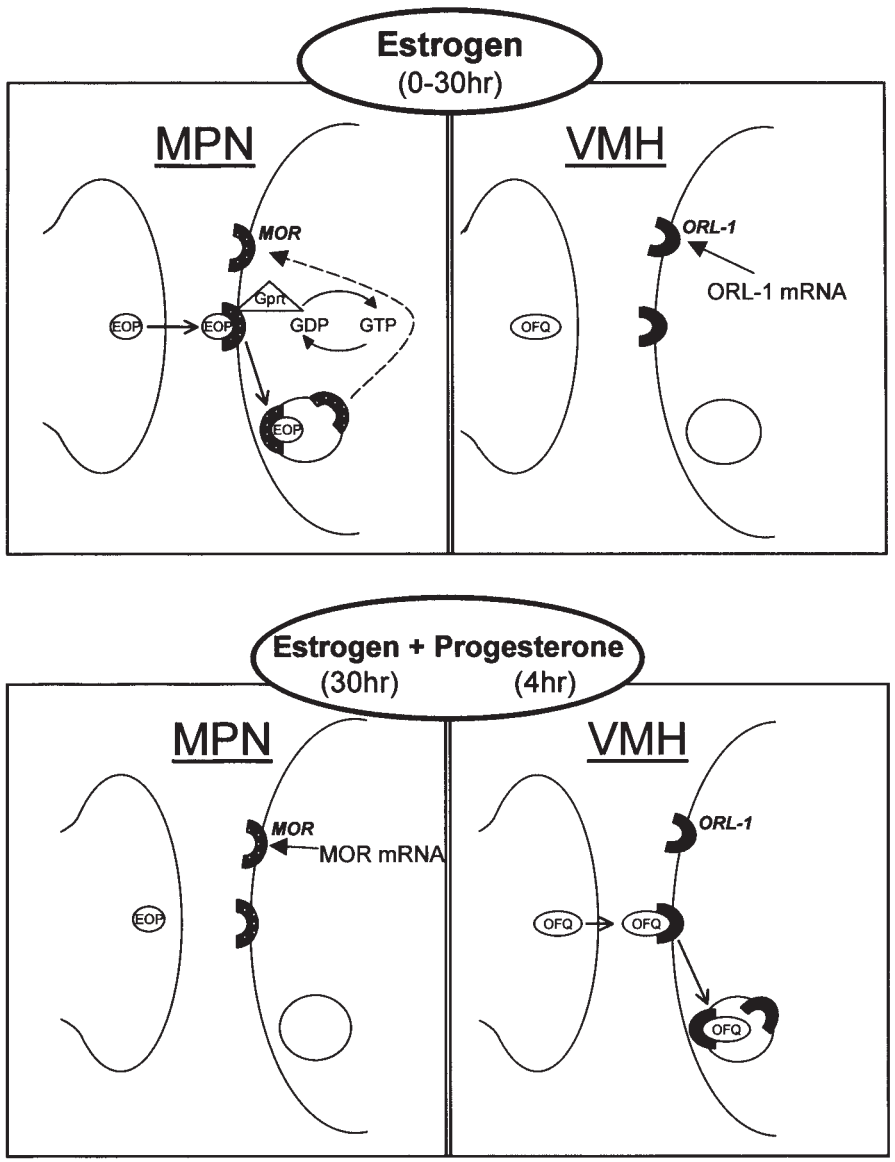

Fig. 11. Schematic of model illustrating the putative interactions of estrogen and progesterone with medial preoptic nucleus (MPN) MOR and ventromedial nucleus of the hypothalamus (VMH) ORL-1 circuits regulating reproductive behavior lordosis. Estrogen increases the efficacy of G protein (Gprt) functional coupling to MOR and induces release of endogenous opioid peptide(s) (EOP) which activates MOR and induces MOR internalization in the MPO. This activation of MOR receptor is very rapid indicating initially that estrogen induces an inhibition of lordosis. Estrogen also initiates the transcription of ORL-1 in the VMH. Subsequent progesterone treatment blocks the release of MOR active EOP in the MPN, allowing MOR to return to the plasma membrane and relieving the inhibitory tone produced by MOR in the MPO. Concurrently in the VMH, progesterone induces the release of OFQ which activates the estrogen induced ORL-1, facilitating lordosis.

Department of Biological Sciences, Brigham Young University, and C. Gray and D. Crandell in the MRRC Media Core, UCLA for their help in preparing the illustrations. This work was supported by NS 21220, NS 39495, and DA13185.

References

1. Dhawan B. N., F. Cesselin, R. Raghubir, T. Reisine, P. B. Bradley, P. S. Portoghese, and M. Hamon. (1996) International union of pharmacology.12. Classification of opioid receptors. *Pharmacological Reviews* **48,** 567–592.
2. Reisine T. and G. I. Bell. (1993) Molecular biology of opioid receptors. *Trends in Neurosciences* **16,** 506–510.
3. Chen Y., A. Mestek, J. Liu, J. A. Hurley, and L. Yu. (1993) Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Molecular Pharmacology* **44,** 8–12.
4. Thompson R. C., A. Mansour, H. Akil, and S. J. Watson. (1993) Cloning and pharmacological characterization of a rat mu-opioid receptor. *Neuron* **11,** 903–913.
5. Wang J. B., P. S. Johnson, A. M. Persico, A. L. Hawkins, C. A. Griffin, and G. R. Uhl. (1994) Human mu opiate receptor. cDNA and genomic clones, pharmacologic characterization and chromosomal assignment. *FEBS Lett.* **338,** 217–222.
6. Mollereau C., M. Parmentier, P. Mailleux, J. L. Butour, C. Moisand, P. Chalon, D. Caput, G. Vassart, and J. C. Meunier. (1994) ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett.* **341,** 33–38.
7. Lachowicz J. E., Y. Shen, F. J. Monsma, Jr., and D. R. Sibley. (1995) Molecular cloning of a novel G protein-coupled receptor related to the opiate receptor family. *J. Neurochem.* **64,** 34–40.
8. Wang J. B., P. S. Johnson, Y. Imai, A. M. Persico, B. A. Ozenberger, C. M. Eppler, and G. R. Uhl. (1994) cDNA cloning of an orphan opiate receptor gene family member and its splice variant. *FEBS Lett.* **348,** 75–79.
9. Zaki P. A., D. E. Keith, J. B. Thomas, F. I. Carroll, and C. J. Evans. (2001) Agonist-, antagonist-, and inverse agonist-regulated trafficking of the delta-opioid receptor correlates with, but does not require, G protein activation. *Journal of Pharmacology and Experimental Therapeutics* **298,** 1015–1020.
10. Zaki P. A., D. E. Keith, G. A. Brine, F. I. Carroll, and C. J. Evans. (2000) Ligand-induced changes in surface mu-opioid receptor number: Relationship to G protein activation? *Journal of Pharmacology and Experimental Therapeutics* **292,** 1127–1134.
11. Daaka Y., L. M. Luttrell, S. Ahn, G. J. DellaRocca, S. S. G. Ferguson, M. G. Caron, and R. J. Lefkowitz. (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *Journal of Biological Chemistry* **273,** 685–688.
12. Luttrell L. M., Y. Daaka, G. J. DellaRocca, and R. J. Lefkowitz. (1997) G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts—Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases. *Journal of Biological Chemistry* **272,** 31,648–31,656.
13. Vieira A. V., C. Lamaze, and S. L. Schmid. (1996) Control of Egf Receptor Signaling by Clathrin-Mediated Endocytosis. *Science* **274,** 2086–2089.
14. Ignatova E. G., M. M. Belcheva, L. M. Bohn, M. C. Neuman, and C. J. Coscia. (1999) Requirement of receptor internalization for opioid stimulation of mitogen-activated protein kinase: Biochemical and immunofluorescence confocal microscopic evidence. *Journal of Neuroscience* **19,** 56–63.
15. Sternini C., N. C. Brecha, J. Minnis, G. D'Agostino, B. Balestra, E. Fiori, and M. Tonini. (2000) Role of agonist-dependent receptor internalization in the regulation of mu opioid receptors. *Neuroscience* **98,** 233–241.
16. Eckersell C. B., P. Popper, and P. E. Micevych. (1998) Estrogen-induced alteration of mu-opioid receptor immunoreactivity in the medial preoptic nucleus and medial amygdala. *J. Neurosci.* **18,** 3967–3976.
17. Sinchak K. and P. E. Micevych. (2001) Progesterone blockade of estrogen activation of μ-opioid receptors regulates reproductive behavior. *J Neurosci.* **21,** 5723–5729.
18. Lefkowitz R. J., S. Cotecchia, M. A. Kjelsberg, J. Pitcher, W. J. Koch, J. Inglese, and M. G. Caron. (1993) Adrenergic receptors: recent insights into their mechanism of activation and desensitization. *Adv. Second Messenger Phosphoprotein Res.* **28,** 1–9.

19. Birnbaumer L. and A. M. Brown. (1990) G proteins and the mechanism of action of hormones, neurotransmitters, and autocrine and paracrine regulatory factors. *Am. Rev. Respir. Dis.* **141,** S106–114.
20. Birnbaumer L., A. Yatani, A. M. VanDongen, R. Graf, J. Codina, K. Okabe, R. Mattera, and A. M. Brown. (1990) G protein coupling of receptors to ionic channels and other effector systems. *Br. J. Clin. Pharmacol.* **30 Suppl 1,** 13S–22S.
21. Rories C. and T. C. Spelsberg. (1989) Ovarian steroid action on gene expression: mechanisms and models. *Annu. Rev. Physiol.* **51,** 653–681.
22. McEwen B. S. (2001) Genome and hormones: Gender differences in physiology – Invited review: Estrogens effects on the brain: multiple sites and molecular mechanisms. *Journal of Applied Physiology* **91,** 2785–2801.
23. Brown E. R., R. E. Harlan, and J. E. Krause. (1990) Gonadal steroid regulation of substance P (SP) and SP-encoding messenger ribonucleic acids in the rat anterior pituitary and hypothalamus. *Endocrinology* **126,** 330–340.
24. Priest C. A., D. Borsook, S. E. Hyman, and D. W. Pfaff. (1995) Estrogen and stress interact to regulate the transcriptional activity of a proenkephalin promoter-beta-GAL fusion gene in the hypothalamus of transgenic mice. *Soc. Neurosci. Abs.* **21,** 1364.
25. Priest C. A., C. B. Eckersell, and P. E. Micevych. (1995) Estrogen regulates preproenkephalin-A mRNA levels in the rat ventromedial nucleus: temporal and cellular aspects. *Brain Res. Mol. Brain Res.* **28,** 251–262.
26. Uht R. M., C. M. Anderson, P. Webb, and P. J. Kushner. (1997) Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. *Endocrinology* **138,** 2900–2908.
27. Akesson T. R. and P. E. Micevych. (1988) Evidence for an absence of estrogen concentration by CCK-immunoreactive neurons in the hypothalamus of the female rat. *J. Neurobiol.* **19,** 3–16.
28. Shivers B. D., R. E. Harlan, J. I. Morrell, and D. W. Pfaff. (1983) Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. *Nature* **304,** 345–347.
29. Watson R. E. (1992) Further evidence that most luteinizing hormone-releasing hormone neurons are not directly estrogen responsive. *J. Neurosci.* **4,** 311–317.
30. Skynner M. J., J. A. Sim, and A. E. Herbison. (2001) Detection of estrogen receptor alpha and beta messenger ribonucleic acids in adult gonadotropin-releasing hormone neurons (vol 140, pg 5195, 1999). *Endocrinology* **142,** 492.
31. Roy D., N. L. Angelini, and D. D. Belsham. (1999) Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ERalpha)- and ERbeta-expressing GT1–7 GnRH neurons. *Endocrinology* **140,** 5045–5053.
32. Nielsen D. A. and Shapiro D. J. (1990) Insights into hormonal control of messenger RNA stability. *Molecular Endocrinology* **4,** 953–957.
33. Singh M., G. Setalo, Jr., X. Guan, D. E. Frail, and C. D. Toran-Allerand. (2000) Estrogen-induced activation of the mitogen-activated protein kinase cascade in the cerebral cortex of estrogen receptor-alpha knock-out mice. *J. Neurosci.* **20,** 1694–1700.
34. Razandi M., A. Pedram, G. Greene, and E. Levin. (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol. Endocrinol.* **13,** 307–319.
35. Razandi M., P. Oh, A. Pedram, J. Schnitzer, and E. R. Levin. (2002) ERs associate with and regulate the production of caveolin: Implications for signaling and cellular actions. *Mol. Endocrinol.* **16,** 100–115.
36. Wade S. B., P. Oommen, W. C. Conner, D. J. Earnest, and R. C. Miranda. (1999) Overlapping and divergent actions of estrogen and the neurotrophins on cell fate and p53-dependent signal transduction in conditionally immortalized cerebral cortical neuroblasts. *J. Neurosci.* **19,** 6994–7006.
37. Toran-Allerand C. D. (1995) Developmental interactions of estrogens with the neurotrophins and their receptors, in *Neurobiological Effects of Sex Steroid Hormones* (Micevych P. E. and Hammer R. P., Jr., eds.), Cambridge University Press, pp. 391–411.
38. Revelli A., M. Massobrio, and J. Tesarik. (1998) Nongenomic actions of steroid hormones in reproductive tissues. *Endocrine Reviews* **19,** 3–17.
39. Chaban V., E. A. Mayer, H. S. Ennes, and P. E. Micevych. Estradiol inhibits ATP-induced $[Ca^{2+}]_i$ increase in DRG. *Neuroscience* (In Press).
40. Moss R. L., Q. Gu, and M. Wong. (1997) Estrogen: nontranscriptional signaling pathway. *Recent Prog. Horm. Res.* **52,** 33–68.

41. Kelly M. and E. Wagner. (1999) Estrogen modulation of G-protein-coupled receptors. *Trends in Endocrinology and Metabolism* **10,** 369–374.
42. Nadal A., M. Diaz, and M. A. Valverde. (2001) The estrogen trinity: Membrane, cytosolic, and nuclear effects. *News in Physiological Sciences* **16,** 251–255.
43. Clemens L. G. and D. R. Weaver. (1985) The role of gonadal hormone in the activation of feminine sexual behavior, in *Handbook of Behavioral Neurobiology* (Adler N., Pfaff D. and Goy R. W., eds.), Plenum Press, New York, pp. 183–227.
44. Bloch G. J., A. M. Babcock, R. A. Gorski, and P. E. Micevych. (1987) Cholecystokinin stimulates and inhibits lordosis behavior in female rats. *Physiol. Behav.* **39,** 217–224.
45. Mani S. K., J. D. Blaustein, and B. W. Omalley. (1997) Progesterone receptor function from a behavioral perspective. *Hormones and Behavior* **31,** 244–255.
46. Blaustein J. D., R. Finkbohner, and Y. Delville. (1987) Estrogen-induced and estrogen-facilitated female rat sexual behavior is not mediated by progestin receptors. *Neuroendocrinology* **45,** 152–159.
47. Quadagno D. M., J. McCullough, and R. Langan. (1972) The effect of varying amounts of exogenous estradiol benzoate on estrous behavior in the rat. *Horm. Behav.* **3,** 175–179.
48. Boling J. L. and R. J. Blandau. (1939) The estrogen-progesterone induction of mating responses in the spayed female rat. *Endocrinology* **25,** 359–364.
49. Sirinathsinghji D. J. S. (1986) Regulation of lordosis behavior in the female rat by corticotropin-releasing factor, beta-endorphin/corticotropin and luteinizing hormone-releasing hormone neuronal systems in the medial preoptic area. *Brain Res.* **375,** 149–156.
50. Pfaus J. G. and D. W. Pfaff. (1992) Mu-, delta-, and kappa-opioid receptor agonists selectively modulate sexual behaviors in the female rat: differential dependence on progesterone. *Horm. Behav.* **26,** 457–473.
51. Sirinathsinghji D. J. (1984) Modulation of lordosis behavior of female rats by naloxone, beta-endorphin and its antiserum in the mesencephalic central gray: possible mediation via GnRH. *Neuroendocrinology* **39,** 222–230.
52. Sirinathsinghji D. J. (1985) Modulation of lordosis behaviour in the female rat by corticotropin releasing factor, beta-endorphin and gonadotropin releasing hormone in the mesencephalic central gray. *Brain Research* **336,** 45–55.
53. Acosta-Martinez M. and A. M. Etgen. (2002) Activation of mu-opioid receptors inhibits lordosis behavior in estrogen and progesterone-primed female rats. *Hormones and Behavior* **41,** 88–100.
54. Wiesner J. B. and R. L. Moss. (1984) Beta-endorphin suppression of lordosis behavior in female rats; lack of effect of peripherally-administered naloxone. *Life Sciences* **34,** 1455–1462.
55. Torii M. and K. Kubo. (1994) The effects of intraventricular injection of beta-endorphin on initial estrogen action to induce lordosis behavior. *Physiol. Behav.* **55,** 157–162.
56. Torii M., K. Kubo, and T. Sasaki. (1995) Naloxone and initial estrogen action to induce lordosis in ovariectomized rats: the effect of a cut between the septum and preoptic area. *Neurosci. Lett.* **195,** 167–170.
57. Torii M., K. Kubo, and T. Sasaki. (1996) Influence of opioid peptides on the priming action of estrogen on lordosis in ovariectomized rats. *Neurosci. Lett.* **212,** 68–70.
58. Mansour A., C. A. Fox, R. C. Thompson, H. Akil, and S. J. Watson. (1994) Mu-opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. *Brain Res.* **643,** 245–265.
59. Mansour A., C. A. Fox, S. Burke, H. Akil, and S. J. Watson. (1995) Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. *J. Chem. Neuroanat.* **8,** 283–305.
60. Pfaff D. W., S. Schwartz-Giblin, M. McCarthy, and L. M. Kow. (1994) Cellular and molecular mechanisms of female reproductive behaviors, in *The Physiology of Reproduction* (Knobil E. and Neill J. D., eds.), Raven Press, Ltd, New York, pp. 107–220.
61. Bunzow J. R., C. Saez, M. Mortrud, C. Bouvier, J. T. Williams, M. Low, and D. K. Grandy. (1994) Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. *FEBS Lett.* **347,** 284–288.
62. Meunier J. C. (1997) Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. *European Journal of Pharmacology* **340,** 1–15.
63. Meunier J. C., C. Mollereau, L. Toll, C. Suaudeau, C. Moisand, P. Alvinerie, J. L.

Butour, J. C. Guillemot, P. Ferrara, B. Monsarrat, et al. (1995) Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor *Nature* **377,** 532–535.
64. Reinscheid R. K., H. P. Nothacker, A. Bourson, A. Ardati, R. A. Henningsen, J. R. Bunzow, D. K. Grandy, H. Langen, F. J. Monsma, Jr., and O. Civelli. (1995) Orphanin FQ: A neuropeptide that activates an opioid-like G protein-coupled receptor. *Science* **270,** 792–794.
65. Sinchak K., D. G. Hendricks, R. Baroudi, and P. E. Micevych. (1997) Orphanin FQ/nociceptin in the ventromedial nucleus facilitates lordosis in female rats. *Neuroreport* **8,** 3857–3860.
66. Darland T., M. M. Heinricher, and D. K. Grandy. (1998) Orphanin FQ/nociceptin: a role in pain and analgesia, but so much more. *Trends in Neuroscience* **21,** 215–221.
67. Sinchak K., H. E. Romeo, and P. E. Micevych. (2001) Estrogen and progestin regulation of OFQ/nociceptin and ORL-1 mRNA expression in the female rat limbic hypothalamic system. *Soc. Neurosci. Abstr.* **27,** 424.4.
68. Lee N. M. and A. P. Smith. (1980) A protein-lipid model of the opiate receptor. *Life Sci.* **26,** 1459–1464.
69. Patterson S. J., L. E. Robson, and H. W. Kosterlitz. (1983) Classification of opiate receptors. *Br. Med. Bull.* **39,** 31–36.
70. Smith A., N. Lee, and H. Loh. (1983) The multiple-site beta-endorphin receptor. *Trends Pharmacol. Sci.* **4,** 163–164.
71. Law P. Y. and H. H. Loh. (1999) Regulation of opioid receptor activities. *Journal of Pharmacology and Experimental Therapeutics* **289,** 607–624.
72. Zadina J. E., L. Hackler, L. J. Ge, and A. J. Kastin. (1997) A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* **386,** 499–502.
73. Harrison C., S. McNulty, D. Smart, D. J. Rowbotham, D. K. Grandy, L. A. Devi, and D. G. Lambert. (1999) The effects of endomorphin-1 and endomorphin-2 in CHO cells expressing recombinant mu-opioid receptors and SH-SY5Y cells. *British Journal of Pharmacology* **128,** 472–478.
74. Finley J. C., P. Lindstrom, and P. Petrusz. (1981) Immunocytochemical localization of beta-endorphin-containing neurons in the rat brain. *Neuroendocrinology* **33,** 28–42.
75. Hammer R. P. and S. Cheung. (1995) Sex steroid regulation of hypothalamic opioid function, in *Neurobiological Effects of Sex Steroid Hormones* (Micevych P. E. and Hammer R. P., eds.), Cambridge University Press, New York, NY, pp. 143–159.
76. Priest C. A. and J. L. Roberts. (2000) Estrogen and tamoxifen differentially regulate beta-endorphin and cFos expression and neuronal colocalization in the arcuate nucleus of the rat. *Neuroendocrinology* **72,** 293–305.
77. Wilcox J. N. and J. L. Roberts. (1985) Estrogen decreases rat hypothalamic proopiomelanocortin messenger ribonucleic acid levels. *Endocrinology* **117,** 2392–2396.
78. Petersen S. L., M. L. Keller, S. A. Carder, and S. McCrone. (1993) Differential Effects of Estrogen and Progesterone on Levels of POMC Messenger RNA Levels in the Arcuate Nucleus—Relationship to the Timing of LH Surge Release. *Journal of Neuroendocrinology* **5,** 643–648.
79. Wise P. M., K. Scarbrough, N. G. Weiland, and G. H. Larson. (1990) Diurnal pattern of proopiomelanocortin gene expression in the arcuate nucleus of proestrous, ovariectomized, and steroid-treated rats: a possible role in cyclic luteinizing hormone secretion. *Mol. Endocrinol.* **4,** 886–892.
80. Cheung S. and R. Hammer. (1995) Gonadal steroid hormone regulation of proopiomelanocortin gene expression in the arcuate neurons that innervate the medial preoptic are of the rat. *Neuroendocrinology* **62,** 283–292.
81. Ge F., R. P. Hammer, Jr., and S. A. Tobet. (1993) Ontogeny of Leu-enkephalin and beta-endorphin innervation of the area in male and female rats. *Brain Res. Dev. Brain Res.* **73,** 273–281.
82. Micevych P. E., C. B. Eckersell, N. Brecha, and K. Holland. (1997) Estrogenic modulation of opiate and cholecystokinin systems in the limbic–hypothalamic circuit. *Brain Res. Bull.* **44,** 335–343.
83. Morrell J. I., J. F. McGinty, and D. W. Pfaff. (1985) A subset of beta-endorphin- or dynorphin-containing neurons in the medial basal hypothalamus accumulates estradiol. *Neuroendocrinology* **41,** 417–426.
84. Jirikowski G. F., I. Merchenthaler, G. E. Rieger, and W. E. Stumpf. (1986) Estradiol target sites immunoreactive for beta-endorphin in the arcuate nucleus of rat and mouse hypothalamus. *Neurosci. Lett.* **65,** 121–126.
85. Simonian S. X., D. P. Spratt, and A. E. Herbison. (1999) Identification and characterization

of estrogen receptor alpha-containing neurons projecting to the vicinity of the gonadotropin-releasing hormone perikarya in the rostral preoptic area of the rat. *Journal of Comparative Neurology* **411,** 346–358.
86. Fodor M. and H. A. Delemarre-van de Waal. (2001) Are POMC neurons targets for sex steroids in the arcuate nucleus of the rat? *Neuroreport* **12,** 3989–3991.
87. Shughrue P. J., M. V. Lane, and I. Merchenthaler. (1997) Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *Journal of Comparative Neurology* **388,** 507–525.
88. Priest C. A., D. Borsook, and D. W. Pfaff. (1997) Estrogen and stress interact to regulate the hypothalamic expression of a human proenkephalin promoter-beta-galactosidase fusion gene in a site-specific and sex-specific manner. *J. Neuroendocrinol.* **9,** 317–326.
89. Sinchak K., C. Eckersell, V. Quezada, A. Norell, and P. Micevych. (2000) Preproenkephalin mRNA levels are regulated by acute stress and estrogen stimulation. *Physiol Behav.* **69,** 425–432.
90. Lopezcalderon A., C. Ariznavarreta, and C. L. C. Chen. (1991) Influence of Chronic Restraint Stress on Proopiomelanocortin Messenger RNA and Beta-Endorphin in the Rat Hypothalamus. *Journal of Molecular Endocrinology* **7,** 197–204.
91. Zhou Y., R. Spangler, C. E. Maggos, X. M. Wang, J. S. Han, A. Ho, and M. J. Kreek. (1999) Hypothalamic-pituitary-adrenal activity and pro-opiomelanocortin mRNA levels in the hypothalamus and pituitary of the rat are differentially modulated by acute intermittent morphine with or without water restriction stress. *Journal of Endocrinology* **163,** 261–267.
92. Martin-Schild S., A. A. Gerall, A. J. Kastin, and J. E. Zadina. (1999) Differential distribution of endomorphin 1- and endomorphin 2-like immunoreactivities in the CNS of the rodent. *Journal of Comparative Neurology* **405,** 450–471.
93. Pierce T. L. and M. W. Wessendorf. (2000) Immunocytochemical mapping of endomorphin-2-immunoreactivity in rat brain. *J. Chem. Neuroanat.* **18,** 181–207.
94. Wang Q. P., J. E. Zadina, J. L. Guan, and S. Shioda. (2002) Morphological studies of the endomorphinergic neurons in the central nervous system. *Japanese Journal of Pharmacology* **89,** 209–215.
95. Zadina J. E. (2002) Isolation and distribution of endomorphins in the central nervous system. *Japanese Journal of Pharmacology* **89,** 203–208.
96. Romano G. J., A. Krust, and D. W. Pfaff. (1989) Expression and estrogen regulation of progesterone receptor mRNA in neurons of the mediobasal hypothalamus: an in situ hybridization study [published erratum appears in Mol. Endocrinol 1989 Aug;3(11):1860]. *Mol. Endocrinol.* **3,** 1295–1300.
97. Romano G. J., R. E. Harlan, B. D. Shiverst, R. D. Howells, and D. W. Pfaff. (1988) Estrogen increases proenkephalin messenger ribonucleic acid levels in the ventromedial hypothalamus of the rat. *Mol. Endocrinol.* **2,** 1320–1328.
98. Holland K., A. Norell, and P. Micevych. (1998) Interaction of thyroxine and estrogen on the expression of estrogen receptor alpha, cholecystokinin, and preproenkephalin messenger ribonucleic acid in the limbic-hypothalamic circuit. *Endocrinology* **139,** 1221–1228.
99. Eckersell C. B., C. A. Priest, and P. E. Micevych. (1994) Temporal regulation of preproenkephalin-A mRNA expression by estrogen in the posterior dorsal medial amygdala of the female rat. *Society for Neuroscience* **20,** 1770.
100. Akesson T. R. and P. E. Micevych. (1991) Endogenous opioid-immunoreactive neurons of the ventromedial hypothalamic nucleus concentrate estrogen in male and female rats. *J. Neurosci. Res.* **28,** 359–366.
101. Reinscheid R. K., J. Higelin, R. A. Henningsen, F. J. Monsma, and O. Civelli. (1998) Structures that delineate orphanin FQ and dynorphin A pharmacological selectivities. *Journal of Biological Chemistry* **273,** 1490–1495.
102. Dooley C. T. and R. A. Houghten. (1996) Orphanin FQ: receptor binding and analog structure activity relationships in rat brain. *Life Sci.* **59,** L23–29.
103. Reinscheid R. K., A. Ardati, F. J. Monsma, and O. Civelli. (1996) Structure-activity relationship studies on the novel neuropeptide orphanin FQ. *Journal of Biological Chemistry* **271,** 14,163–14,168.
104. Shimohigashi Y., R. Hatano, T. Fujita, R. Nakashima, T. Nose, T. Sujaku, A. Saigo, K. Shinjo, and A. Nagahisa. (1996) Sensitivity of opioid receptor-like receptor ORL1 for chemical modification on nociceptin, a naturally occurring nociceptive peptide. *J. Biol. Chem.* **271,** 23,642–23,645.

105. Ardati A., R. A. Henningsen, J. Higelin, R. K. Reinscheid, O. Civelli, and F. J. Monsma. (1997) Interaction of [H-3]orphanin FQ and I-125-Tyr14-orphanin FQ with the orphanin FQ receptor: Kinetics and modulation by cations and guanine nucleotides. *Molecular Pharmacology* **51,** 816–824.
106. Butour J. L., C. Moisand, H. Mazarguil, C. Mollereau, and J. C. Meunier. (1997) Recognition and activation of the opioid receptor-like ORL 1 receptor by nociceptin, nociceptin analogs and opioids. *European Journal of Pharmacology* **321,** 97–103.
107. Guerrini R., G. Calo, A. Rizzi, C. Bianchi, L. H. Lazarus, S. Salvadori, P. A. Temussi, and D. Regoli. (1997) Address and message sequences for the nociceptin receptor: A structure-activity study of nociceptin-(1–13)-peptide amide. *Journal of Medicinal Chemistry* **40,** 1789–1793.
108. Anton B., J. Fein, T. To, X. Li, L. Silberstein, and C. J. Evans. (1996) Immunohistochemical localization of ORL-1 in the central nervous system of the rat. *J. Comp. Neurol.* **368,** 229–251.
109. Chen Y., Y. Fan, J. Liu, A. Mestek, M. Tian, C. A. Kozak, and L. Yu. (1994) Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family. *FEBS Lett.* **347,** 279–283.
110. Fukuda K., S. Kato, K. Mori, M. Nishi, H. Takeshima, N. Iwabe, T. Miyata, T. Houtani, and T. Sugimoto. (1994) cDNA cloning and regional distribution of a novel member of the opioid receptor family. *FEBS Lett.* **343,** 42–46.
111. Riedl M., S. Shuster, L. Vulchanova, J. Wang, H. H. Loh, and R. Elde. (1996) Orphanin FQ/nociceptin-immunoreactive nerve fibers parallel those containing endogenous opioids in rat spinal cord. *Neuroreport* **7,** 1369–1372.
112. Wick M. J., S. R. Minnerath, X. Lin, R. Elde, P. Y. Law, and H. H. Loh. (1994) Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned mu, delta, and kappa opioid receptors. *Brain Res. Mol. Brain Res.* **27,** 37–44.
113. Lee K., J. R. Nicholson, and A. T. McKnight. (1997) Nociceptin hyperpolarises neurones in the rat ventromedial hypothalamus. *Neuroscience Letters* **239,** 37–40.
114. Martini L., D. Dondi, P. Limonta, R. Maggi, and F. Piva. (1989) Modulation by sex steroids of brain opioid receptors: implications for the control of gonadotropins and prolactin secretion. *J. Steroid Biochem.* **33,** 673–681.
115. Mateo A. R., M. Hijazi, and R. P. Hammer, Jr. (1992) Dynamic patterns of medial preoptic mu-opiate receptor regulation by gonadal steroid hormones. *Neuroendocrinology* **55,** 51–58.
116. Thom B., B. J. Canny, M. Cowley, P. J. Wright, and I. J. Clarke. (1996) changes in the binding characteristics of the μ, δ, and κ subtypes of the opioid receptor in the hypothalamus of the normal cyclic ewe and in the ovariectomized ewe following treatment with ovarian steroids. *J. Endocrinol.* **149,** 509–518.
117. Weiland N. G. and P. M. Wise. (1990) Estrogen and progesterone regulate opiate receptor densities in multiple brain regions. *Endocrinology* **126,** 804–808.
118. Wilkinson M., J. R. Brawer, and D. A. Wilkinson. (1985) Gonadal steroid-induced modification of opiate binding sites in anterior hypothalamus of female rats. *Biol. Reprod.* **32,** 501–506.
119. Zhou L. and R. P. Hammer, Jr. (1995) Gonadal steroid hormones upregulate medial preoptic mu-opioid receptors in the rat. *Eur. J. Pharmacol.* **278,** 271–274.
120. Piva F., P. Limonta, D. Dondi, F. Pimpinelli, L. Martini, and R. Maggi. (1995) Effects of steroids on the brain opioid system. *J. Steroid Biochem. Mol. Biol.* **53,** 343–348.
121. Maggi R., D. Dondi, G. E. Rovati, L. Martini, F. Piva, and P. Limonta. (1993) Binding Characteristics of Hypothalamic Mu Opioid Receptors Throughout the Estrous Cycle in the Rat. *Neuroendocrinology* **58,** 366–372.
122. Micevych P. E., V. Chaban, A. Quesada, and K. Sinchak. (2002) Estrogen modulates CCK—Opioid interactions in the nervous system. *Pharmacology and Toxicology* **91,** 387–397.
123. Allen B. J., S. D. Rogers, J. R. Ghilardi, P. M. Menning, M. A. Kuskowski, A. I. Basbaum, D. A. Simone, and P. W. Mantyh. (1997) Noxious cutaneous thermal stimuli induce a graded release of endogenous substance P in the spinal cord: Imaging peptide action in vivo. *J. Neurosci.* **17,** 5921–5927.
124. Grady E. F., A. M. Garland, P. D. Gamp, M. Lovett, D. G. Payan, and N. W. Bunnett. (1995) Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. *Mol. Biol. Cell.* **6,** 509–524.
125. Hoxie J. A., M. Ahuja, E. Belmonte, S. Pizarro, R. Parton, and L. F. Brass. (1993) Internalization and recycling of activated thrombin receptors. *J. Biol. Chem.* **268,** 13,756–13,763.

126. Parker E. M., P. Swigart, M. H. Nunnally, J. P. Perkins, and E. M. Ross. (1995) Carboxyl-terminal domains in the avian beta 1-adrenergic receptor that regulate agonist-promoted endocytosis [published erratum appears in J. Biol. Chem. 1995 Apr 28;270(17):10358]. *J. Biol. Chem.* **270,** 6482–6487.
127. von Zastrow M. and B. K. Kobilka. (1992) Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.* **267,** 3530–3538.
128. Caron M. G. and R. J. Lefkowitz. (1993) Catecholamine receptors: Structure, function, and regulation. *Recent Prog. Horm. Res.* **48,** 277–290.
129. Mantyh P. W., C. J. Allen, J. R. Ghilardi, S. D. Rogers, C. R. Mantyh, H. Liu, A. I. Basbaum, S. R. Vigna, and J. E. Maggio. (1995) Rapid endocytosis of a G protein-coupled receptor: Substance P evoked internalization of its receptor in the rat striatum in vivo. *Proc. Natl. Acad. Sci. USA* **92,** 2622–2626.
130. Mantyh P. W., E. DeMaster, A. Malhotra, J. R. Ghilardi, S. D. Rogers, C. R. Mantyh, H. Liu, A. I. Basbaum, S. R. Vigna, J. E. Maggio, et al. (1995) Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science* **268,** 1629–1632.
131. Sternini C., M. Spann, B. Anton, D. E. Keith, Jr., N. W. Bunnett, M. von Zastrow, C. Evans, and N. C. Brecha. (1996) Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. *Proc. Natl. Acad. Sci. USA* **93,** 9241–9246.
132. von Zastrow M., D. Keith, P. Zaki, and C. Evans. (1994) Intracellular trafficking of epitope-tagged opioid receptors different effects of morphine and enkephalin. *Regul. Pept.* **54,** 315–316.
133. Law P. Y., D. S. Hom, and H. H. Loh. (1982) Loss of opiate receptor activity in neuroblastoma X glioma NG108–15 hybrid cells after chronic opiate treatment. A multiple-step process. *Molecular Pharmacology* **22,** 1–4.
134. Law P. Y., D. S. Hom, and H. H. Loh. (1984) Down-regulation of opiate receptor in neuroblastoma x glioma NG108–15 hybrid cells. Chloroquine promotes accumulation of tritiated enkephalin in the lysosomes. *Journal of Biological Chemistry* **259,** 4096–4104.
135. Sharma S. K., W. A. Klee, and M. Nirenberg. (1977) Opiate-dependent modulation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **74,** 3365–3369.
136. Mestek A., J. H. Hurley, L. S. Bye, A. D. Campbell, Y. Chen, M. Tian, J. Liu, H. Schulman, and L. Yu. (1995) The human mu opioid receptor. *Journal of Neuroscience* **15,** 2396–2406.
137. Louie A. K., J. N. Zhan, P. Y. Law, and H. H. Loh. (1988) Modification of opioid receptor activity by acid phosphatase in neuroblastoma x glioma NG108–15 hybrid cells. *Biochemical and Biophysical Research Communications* **152,** 1369–1375.
138. Arden J. R., V. Segredo, Z. Wang, J. Lameh, and W. Sadaee. (1995) Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-opioid receptor expressed in HEK 293 cells. *Journal of Neurochemistry* **65,** 1636–1645.
139. Keith D. E., S. R. Murray, P. A. Zaki, P. C. Chu, D. V. Lissin, L. Kang, C. J. Evans, and M. von Zastrow. (1996) Morphine activates opioid receptors without causing their rapid internalization. *J. Bio. Chem.* **271,** 19,021–19,024.
140. von Zastrow M., D. E. Keith, Jr., and C. J. Evans. (1993) Agonist-induced state of the delta-opioid receptor that discriminates between opioid peptides and opiate alkaloids. *Mol. Pharmacol.* **44,** 166–172.
141. Eckersell C. B. and P. E. Micevych (submitted) Distribution of μ- and δ-opioid receptors with respect to CCK immunoreactivity in the hypothalamus and limbic system.
142. Sherman M. R. and S. C. Diaz. (1977) Meroreceptor formation from a larger subcomponent of the oviduct progesterone receptor. *Ann. NY Acad. Sci.* **286,** 81–86.
143. Olive F. M., J. C. Evans, P. E. Micevych, and N. T. Maidment. (1997) Pre- vs Postsynaptic localization of mu and delta opioid receptors in the striatopallidal pathway. *J. Neurosci.* **17,** 7471–7479.
144. Arvidsson U., M. Riedl, S. Chakrabarti, J. H. Lee, A. H. Nakano, R. J. Dado, H. H. Loh, P. Y. Law, M. W. Wessendorf, and R. Elde. (1995) Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. *J. Neurosci.* **15,** 3328–3341.
145. Elde R., U. Arvidsson, M. Riedl, L. Vulchanova, J. H. Lee, R. Dado, A. Nakano, S. Chakrabarti, X. Zhang, H. H. Loh, et al. (1995) Distribution of neuropeptide receptors. New views of peptidergic neurotransmission made possible by antibodies to opioid receptors. *Ann. NY Acad. Sci.* **757,** 390–404.

146. Cheng P. Y., L. Y. Liu-Chen, C. Chen, and V. M. Pickel. (1996) Immunolabeling of Mu opioid receptors in the rat nucleus of the solitary tract: extrasynaptic plasmalemmal localization and association with Leu5-enkephalin. *J. Comp. Neurol.* **371,** 522–536.
147. Svingos A. L., A. Moriwaki, J. B. Wang, G. R. Uhl, and V. M. Pickel. (1996) Ultrastructural immunocytochemical localization of mu-opioid receptors rat nucleus accumbens: extrasynaptic plasmalemmal distribution and association with Leu5-enkephalin. *J. Neurosci.* **16,** 4162–4173.
148. Wang X. M., K. M. Zhang, and S. S. Mokha. (1996) Nociceptin (orphanin FQ), an endogenous ligand for the ORL1 (opioid-receptor-like1) receptor; modulates responses of trigeminal neurons evoked by excitatory amino acids and somatosensory stimuli. *J. Neurophysiol.* **76,** 3568–3572.
149. Van Bockstaele E. J., E. E. Colago, A. Moriwaki, and G. R. Uhl. (1996) Mu-opioid receptor is located on the plasma membrane of dendrites that receive asymmetric synapses from axon terminals containing leucine-enkephalin in the rat nucleus locus coeruleus. *J. Comp. Neurol.* **376,** 65–74.
150. Keith D. E., B. Anton, S. R. Murray, P. A. Zaki, P. C. Chu, D. V. Lissin, G. Monteillet-Agius, P. L. Stewart, C. J. Evans, and M. von Zastrow. (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Molecular Pharmacology* **53,** 377–384.
151. McConalogue K., E. F. Grady, J. Minnis, B. Balestra, M. Tonini, N. C. Brecha, N. W. Bunnett, and C. Sternini. (1999) Activation and internalization of the mu-opioid receptor by the newly discovered endogenous agonists, endomorphin-1 and endomorphin-2. *Neuroscience* **90,** 1051–1059.
152. Keith D., S. Murray, P. Zaki, P. Chu, D. Lisson, L. Kang, J. Aimi, C. Evans, and M. von Zastrow. (1995) Rapid endocytosis of opioid receptors: Differential regulation by opioid peptide and morphine. *Soc. Neurosci. Abstr.* **21,** 1353.
153. Dournaud P., H. Boudin, A. Schonbrunn, G. S. Tannenbaum, and A. Beaudet. (1998) Interrelationships between somatostatin sst2A receptors and somatostatin-containing axons in rat brain: Evidence for regulation of cell surface receptors by endogenous somatostatin. *Journal of Neuroscience* **18,** 1056–1071.
154. Jacobson W. and S. P. Kalra. (1989) Decreases in mediobasal hypothalamic and preoptic area opioid ([3H]naloxone) binding are associated with the progesterone-induced luteinizing hormone surge. *Endocrinology* **124,** 199–206.
155. Zhou Y., Y.-H. Sun, Z.-W. Zhang, and J.-S. Han. (1993) Increased release of immunoreactive cholecystokinin octapeptide by morphine and potentiation of mu-opioid analgesia by CCK-B receptor antagonist L-365,260 in rat spinal cord. *Europ. J. Pharmoc.* **234,** 147–154.
156. Collin E., S. Bourgoin, S. Mantelet, M. Hamon, and F. Cesselin. (1992) Feedback inhibition of met-enkephalin release from the rat spinal cord in vivo. *Synapse* **11,** 76–84.
157. Pierce T. L. and M. W. Wessendorf. (2000) Immunocytochemical mapping of endomorphin-2-immunoreactivity in rat brain. *Journal of Chemical Neuroanatomy* **18,** 181–207.
158. Shughrue P. J., M. V. Lane, P. J. Scrimo, and I. Merchenthaler. (1998) Comparative distribution of estrogen receptor-alpha (ER-alpha) and beta (ER-beta) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* **63,** 498–504.
159. Clark J. T., P. S. Kalra, and S. P. Kalra. (1985) Neuropeptide Y stimulates feeding but inhibits sexual behavior in rats. *Endocrinology* **117,** 2435–2442.
160. Mills R. H., R. K. Sohn, and P. E. Micevych. (2001) Effects of neuropeptide Y on steroid activation of m-opioid receptor and neuropeptide Y-Y1 receptor in the hypothalamus in female rats. *Society for Behavioral Neuroendocrinology* **39,** 339–340.
161. Webb P., G. N. Lopez, R. M. Uht, and P. J. Kushner. (1995) Tamoxifen activation of the estrogen. *Molecular Endocrinology* **9,** 443–456.
162. Gu Q., K. S. Korach, and R. L. Moss. (1999) Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. *Endocrinology* **140,** 660–666.
163. Lubahn D. B., J. S. Moyer, T. S. Golding, J. F. Couse, K. S. Korach, and O. Smithies. (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. USA* **90,** 11,162–11,166.
164. Korach K. S.. (1994) Insights from the study of animals lacking functional estrogen receptor. *Science* **266,** 1524–1527.

165. Kuiper G., E. Enmark, M. Pelto-Huikko, S. Nilsson, and J.-A. Gustafsson. (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci.* **93,** 5925–5930.
166. Kuiper G. G., P. J. Shughrue, I. Merchenthaler, and J. A. Gustafsson. (1998) The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems. *Frontiers in Neuroendocrinology* **19,** 253–286.
167. Kuiper G. G., B. Carlsson, K. Grandien, E. Enmark, J. Heaggblad, S. Nilsson, and J. A. Gustafsson. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138,** 863–870.
168. Rissman E. F., S. R. Wersinger, J. A. Taylor, and D. B. Lubahn. (1997) Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects. *Hormones and Behavior* **31,** 232–243.
169. Tremblay G. B., A. Tremblay, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, F. Labrie, and V. Giguere. (1997) Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol. Endocrinol.* **11,** 353–365.
170. Mosselman S., J. Polman, and R. Dijkema. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **392,** 49–53.
171. Micevych P. E., E. F. Rissman, and K. Sinchak. (2003) Estrogen receptor-α is required for estrogen-induced μ-opioid receptor internalization. *J. Neurosci. Res.* **71,** 802–810.
172. Fedynyshyn J. P. and N. M. Lee. (1989) Mu-type opioid receptors in rat periaqueductal gray-enriched P2 membrane are coupled to guanine nucleotide binding proteins. *Brain Res.* **476,** 102–109.
173. Selley D. E. and J. M. Bidlack. (1992) Effects of Beta-Endorphin on Mu and Delta Opioid Receptor-Coupled G-Protein Activity—Low-Km GTPase Studies. *Journal of Pharmacology and Experimental Therapeutics* **263,** 99–104.
174. Chakrabarti S., P. L. Prather, L. Yu, P. Y. Law, and H. H. Loh. (1995) Expression of the mu-opioid receptor in CHO cells—Ability of mu-opioid ligands to promote alpha-azidoanilido [P-32]GTP labeling of multiple G protein alpha subunits. *J. Neurochem.* **64,** 2534–2543.
175. Frey E. A. and J. W. Kebabian. (1984) A mu-opiate receptor in 7315c tumor tissue mediates inhibition of immunoreactive prolactin release and adenylate cyclase activity. *Endocrinology* **115,** 1797–1804.
176. Yu V. C. and W. Sadee. (1988) Efficacy and tolerance of narcotic analgesics at the mu opioid receptor in differentiated human neuroblastoma cells. *J. Pharmacol. Exp. Ther.* **245,** 350–355.
177. Childers S. R.. (1991) Opioid receptor-coupled second messenger systems. *Life Sci.* **48,** 1991–2003.
178. Aghajanian G. K. and Y. Y. Wang. (1986) Pertussis toxin blocks the outward currents evoked by opiate and alpha 2-agonists in locus coeruleus neurons. *Brain Res.* **371,** 390–394.
179. North R. A., J. T. Williams, A. Surprenant, and M. J. Christie. (1987) Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc. Natl. Acad. Sci. USA* **84,** 5487–5491.
180. Moises H. C., K. I. Rusin, and R. L. Macdonald. (1994) Mu- and kappa-opioid receptors selectively reduce the same transient components of high-threshold calcium current in rat dorsal root ganglion sensory neurons. *J. Neurosci.* **14,** 5903–5916.
181. Moises H. C., K. I. Rusin, and R. L. Macdonald. (1994) mu-Opioid receptor-mediated reduction of neuronal calcium current occurs via a G(o)-type GTP-binding protein. *J. Neurosci.* **14,** 3842–3851.
182. Rhim H. and R. J. Miller. (1994) Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius of the rat. *J. Neurosci.* **14,** 7608–7615.
183. Kelly M. J. and E. R. Levin. (2001) Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol. Metab.* **12,** 152–156.
184. Kelly M. J., A. H. Lagrange, E. J. Wagner, and O. K. Ronnekleiv. (1999) Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. *Steroids* **64,** 64–75.
185. Russell K. S., M. P. Haynes, D. Sinha, E. Clerisme, and J. R. Bender. (2000) Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc. Nat. Acad. Sci. USA* **97,** 5930–5935.
186. Pietras R. J., I. Nemere, and C. M. Szego. (2001) Steroid hormone receptors in target cell membranes. *Endocrine* **14,** 417–427.
187. Pietras R. J. and C. M. Szego. (1999) Cell membrane estrogen receptors resurface. *Nat. Med.* **5,** 1330.

188. Pietras R. J. and C. M. Szego. (1977) Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* **265,** 69–72.
189. Sim L. J. and S. R. Childers. (1997) Anatomical distribution of mu, delta, and kappa opioid- and nociceptin/orphanin FQ stimulated [^{35}S]Guanylyl-5′-*O*-(γ-thio)-triphosphate binding in guinea pig brain. *J. Comp. Neurol.* **386,** 562–572.
190. Sim L. J., Q. Liu, S. R. Childers, and D. E. Selley. (1998) Endomorphin-stimulated [35S]GTPgammaS binding in rat brain: evidence for partial agonist activity at mu-opioid receptors. *Journal of Neurochemistry* **70,** 1567–1576.
191. Sim L. J., D. E. Selley, and S. R. Childers. (1995) In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[gamma-[35S]thio]-triphosphate binding. *Proc. Nat. Acad. Sci. USA* **92,** 7242–7246.
192. Sim L. J., D. E. Selley, S. I. Dworkin, and S. R. Childers. (1996) Effects of chronic morphine administration on μ-opioid receptor-stimulated [35S]GTPγS autoradiography in rat brain. *J. Neurosci.* **16,** 2684–2692.
193. Sim L. J., D. E. Selley, R. Xiao, and S. R. Childers. (1996) Differences in G-protein activation by μ- and δ-opioid, and cannabinoid, receptors in rat striatum. *Europ. J. Pharmacol.* **307,** 97–105.
194. Selley D. E., L. J. Sim, R. Y. Xiao, Q. X. Liu, and S. R. Childers. (1997) mu-Opioid receptor-stimulated guanosine-5′-*O*-(γ-thio)-triphosphate binding in rat thalamus and cultured cell lines: Signal transduction mechanisms underlying agonist efficacy. *Mol. Pharmacol.* **51,** 87–96.
195. Cunningham M. J., Y. Fang, D. E. Selley, and M. J. Kelly. (1998) mu-Opioid agonist-stimulated [35S]GTPγS binding in guinea pig hypothalamus: effects of estrogen. *Brain Res.* **791,** 341–346.
196. Bueno J. and D. W. Pfaff. (1976) Single unit recording in hypothalamus and preoptic area of estrogen-treated and untreated ovariectomized female rats. *Brain Res.* **101,** 67–78.
197. Lincoln D. W. (1967) Unit activity in the hypothalamus, septum and preoptic area of the rat: characteristics of spontaneous activity and the effect of oestrogen. *J. Endocrinol.* **37,** 177–189.
198. Sinchak K., V. Yu, and P. E. Micevych. (1998) Progesterone induces internalization of ORl-1 in the ventromedial nucleus of the hypothalamus in estrogen primed female rats but does not increase the area of immunoreactivity. *Soc. Neurosci. Abstr.* **24,** 1011.
199. Neal C. R., A. Mansour, R. Reinscheid, H. P. Nothacker, O. Civelli, H. Akil, and S. J. Watson. (1999) Opioid receptor-like (ORL1) receptor distribution in the rat central nervous system: Comparison of ORL1 receptor mRNA expression with I-125-[(14)Tyr]-orphanin FQ binding. *J. Comp. Neurol.* **412,** 563–605.
200. Sinchak K., R. Khavari, D. Katzman, and P. E. Micevych. (1999) Sexual receptivity is associated with a reduction in internalization of mu-opioid receptors in the medial preoptic nucleus in cycling and steroid primed ovariectomized rats. *Soc. Neurosci. Abst.* **25,** 1705.
201. Popper P., C. A. Priest, and P. E. Micevych. (1996) Regulation of cholecystokinin receptors in the ventromedial nucleus of the hypothalamus: Sex steroid hormone regulation. *Brain Res.* **715,** 335–339.