ELSEVIER

European Journal of Pharmacology 352 (1998) 73–79

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The role of endogenous opioids in the luteinizing hormone surge in rats: studies with clocinnamox, a long-lasting opioid receptor antagonist

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Received 17 November 1997; revised 22 April 1998; accepted 28 April 1998

Abstract

Endogenous opioid peptides have been demonstrated to regulate luteinizing hormone (LH) secretion in a variety of species. Studies in rodents suggest a role of opioid peptide systems in controlling the timing of the LH surge, which is entrained to the circadian rhythm. The current studies utilize clocinnamox, a novel long-lasting opioid receptor antagonist that is capable of occupying μ-opioid receptors for periods of one week or more, to examine the role of endogenous opioid systems on the LH surge. Administration of clocinnamox [14b-(*p*-chlorocinamoylamino)-7,8-dihydro-*N*-cyclopropylmethyl normophineone mesylate]) on the morning of proestrus advanced the LH surge by several hours. Despite the blockade of opioid receptors and analgesia for more than one week, administration of clocinnamox on the evening of diestrus II had no effect on the timing of the LH surge but significantly increased plasma LH levels throughout the day of proestrus. These data suggest that removal of opioid tone is unlikely to be the critical signal controlling the initiation of the LH surge in rodents, although it does appear to be permissive for the surge. Furthermore, the μ-opioid receptor appears to be the receptor involved in the regulation of the LH surge.

Keywords: μ-Opioid receptor; Reproductive hormone; β-Endorphin

1. Introduction

Opiates have long been known to modulate the reproductive axis. Acute administration of morphine on the afternoon of proestrus can block the luteinizing hormone (LH) surge (Barraclough and Sawyer, 1955). Administration of naloxone, a relatively short-acting, non-specific opioid receptor antagonist given on the morning of proestrus can advance the LH surge in rats (Allen and Kalra, 1986). The above data support the prevailing model that a decrease in opioid tone on the day of proestrus is the trigger for the LH surge (Piva et al., 1985). In addition to advancing the LH surge, acute injections of naloxone appear to increase plasma LH levels throughout the estrous cycle (Allen and Kalra, 1986; Allen et al., 1988), leading to the hypothesis that there is opioid tone throughout the estrous cycle except for the afternoon of proestrus.

Of the various opioid peptides, β-endorphin appears to be the peptide most strongly implicated in the regulation of LH secretion (Kalra, 1993). Arcuate β-endorphin neurons have estrogen receptors and their terminals synapse upon gonadotropin-releasing-hormone (GnRH) neurons, providing neuroanatomical evidence of opioid input to GnRH secretion (Morrell et al., 1985; Leranth et al., 1988). Furthermore, ovarian steroids have been demonstrated to regulate β-endorphin peptide levels and proopiomelanocortin (POMC, the β-endorphin precursor) mRNA levels (Wardlaw et al., 1982; Treiser and Wardlaw, 1992; Peterson et al., 1993). Decreases in β-endorphin in the hypophyseal portal blood, in hypothalamic β-endorphin stores (Sarkar and Minami, 1995; Barden et al., 1981), in POMC mRNA, and in POMC primary transcript (Scarbrough et al., 1994; Wise et al., 1990; Bohler et al., 1991) have been found on the afternoon of proestrus, lending further support for a role of β-endorphin in the regulation of the LH surge.

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0014-2999/98/$19.00

β-Endorphin binds to both μ- and δ-opioid receptors and which of these opioid receptors is the critical receptor is unclear. Pharmacological studies have generally used naloxone, which exerts inhibitory effects at multiple opioid receptors (Barraclough and Sawyer, 1955; Allen and Kalra, 1986; Piva et al., 1985; Allen et al., 1988). However, evidence exists that δ-opioid receptors (Wiesner et al., 1985) μ_1-opioid receptors (Rivest et al., 1993) and κ-opioid receptors (Zhen and Gallo, 1992) regulate LH secretion in different models. Given the evidence supporting the involvement of multiple opioid receptors under different experimental conditions, the following experiments using clocinnamox were undertaken to determine whether a release from endogenous opioid tone at the μ-opioid receptor is the critical signal that initiates the normal LH surge in rats.

Clocinnamox [14b-(*p*-chlorocinamoylamino)-7,8-dihydro-*N*-cyclopropylmethyl normophineone mesylate]), was first reported by Aceto et al. (1989) and synthesized by Lewis et al. (1989). Clocinnamox is a long-acting and specific μ-opioid receptor antagonist (Aceto et al., 1989; Paronis and Woods, 1997). In vivo, it has been shown to reduce the analgesic effects of morphine in rhesus monkeys, mice, and rats for periods exceeding 1 week, without showing any agonist activity (Butelman et al., 1996; Chan et al., 1995). Because of its ability to block μ-opioid receptors long-term, clocinnamox provides long-lasting functional removal of opioid activity at the μ-opioid receptor. Clocinnamox can be administered by a subcutaneous injection, reducing the stress involved in administration of the opioid receptor antagonist. Furthermore, its long duration of action enables varying the time between the removal of opioid tone and the proestrus LH surge. This allows us to ascertain whether a decrease in opioid tone is the trigger that initiates the LH surge, or if it is a permissive condition for the occurrence of the LH surge. Finally, because of its greater selectivity for the μ-opioid receptor, clocinnamox allows us to address the specific involvement of μ-opioid receptors in the regulation of LH.

2. Materials and methods

2.1. Animals

Adult female Sprague–Dawley rats obtained from the Reproductive Sciences Program rodent core were maintained in a temperature-controlled environment with lights on from 05:00–19:00. Food and water were available ad libitum. Rats were group-housed three per cage and vaginally smeared daily to determine the stage of cycle throughout the duration of the experiments. Rats with regular 4- or 5-day estrus cycles were used for these studies. In all experiments, blood was collected by tail-nick, a small incision in the tail vein made with a razor blade. At each time point, 400–500 μl of blood was taken. Blood was collected into an Eppendorf tube containing 50 μl of 20 mM EDTA. Plasma was separated by centrifugation and frozen at −20°C until assayed.

2.2. Measurement of plasma LH

LH concentrations were determined by radioimmunoassay according to the method of Niswender designed to measure LH in sheep (Niswender et al., 1969) and adapted for measurement of rat LH (Niswender et al., 1968). No. 15 LH antiserum was obtained from the Assay and Reagent Core of the Reproductive Sciences Program and used at a dilution of 1:300,000 in 0.1% gel-phosphate-buffered saline (assay buffer). [^{125}I]ovine LH, radioiodinated from an ovine LH preparation supplied by the National Institute of Diabetes and Digestive and Kidney Diseases, was used as radiolabelled tracer, and results were expressed as ng of rat RP-1-standard/ml (from the National Institute of Diabetes and Digestive and Kidney Diseases). The standard curve was serial 1:2 dilutions of rat RP-1 ranging from 3.3–845 ng/ml. Total assay volume was 250 μl, containing 100 μl of sample or standard, 100 μl of no. 15 LH antiserum, and 50 μl radiolabelled tracer. In cases where LH levels were expected to be low, 100 μl of sample was used, while during the LH surge, 5 μl of sample was diluted in assay buffer to a volume of 100 μl for the assay. The assay was performed at disequilibrium conditions. Second antibody (goat-anti-rabbit) was used to precipitate [^{125}I]oLH bound to the primary antibody. Samples were assayed in duplicate. Assay sensitivity allowed detection of LH levels for as low as 10 ng/ml. Interassay variability was 11.1% and intraassay variability was 9.2%.

2.3. Analgesia testing: tail-withdrawal latency

Rats were held in a restrainer with tails accessible. After a 20-s habituation period in room temperature water, tails were dried and dipped in 55°C water. Latency to tail-withdrawal was recorded with a maximal cutoff time being 20 s in all cases. After another 20-s habituation period, the tail-withdrawal procedure was repeated. These two values were averaged to determine baseline tail-withdrawal latency. After injection of morphine (20–100 mg/kg, s.c.), tail-withdrawal latency was repeated 20–25 min later.

2.4. Data analyses

Most experiments utilized a repeated measure paradigm and thus were analyzed using a repeated measures analysis of variance (ANOVA). Plasma LH values were log-transformed prior to statistical analyses using one- or two-way ANOVA. Effects were termed significant if $P < 0.05$. Post-hoc testing (Scheffe) was used for specific contrasts within an experiment.

2.5. Experimental design

2.5.1. Experiment no. 1: validation that clocinnamox can block a known opioid receptor effect on the reproductive axis

Previous studies by us (Paronis and Woods, 1997) in intact female rats validated that clocinnamox produced blockade of morphine analgesia and maximal occupation of opioid receptors by 2 h. To verify that clocinnamox would block a known opioid effect on the LH surge mechanism, the ability of clocinnamox to antagonize morphine-induced suppression of ovulation was then evaluated. We chose morphine because previous studies have demonstrated that high-dose morphine could block the LH surge in rats. However, the opioid receptor type involved in this effect of morphine is unclear. Because clocinnamox is an essentially irreversible drug, it is impossible to randomize treatment while using each rat as its own control. Thus, plasma LH levels were compared in two consecutive cycles in seven untreated female rats at 08:30 and 16:30 of proestrus and found not to differ significantly between cycles. Because the dose of morphine needed to suppress ovulation is quite large and near the LD_{50}, a multi-step design was used to minimize respiratory depression. After an initial estrous cycle where blood was taken from rats during the LH surge (16:30 proestrus), rats were vehicle-injected on the night before the LH surge and then injected with morphine, 20 mg/kg, i.p. at 13:30 on the subsequent day, proestrus (Cycle 2, see Fig. 1). Blood was taken at 16:30 on the same day. Blood samples were assayed for LH to determine if 20 mg/kg of morphine could block the LH surge. If 20 mg/kg did not block the LH surge in a particular rat, then 32 mg/kg morphine was injected at 13:30 on the day of proestrus of the subsequent cycle. After the successful blockade of the LH surge in all rats, rats were allowed to cycle normally through a rest cycle (third cycle) and then blood was taken on fourth cycle to determine that morphine had no long-term effects on the magnitude or timing of the LH surge. On the fifth estrous cycle, clocinnamox was injected at 17:30 on diestrus II, the day before the LH surge. Rats then received the same dose of morphine as in the previous morphine cycle (20 or 32 mg/kg) at 13:30 on the day of proestrus. Blood was taken at 16:30 to determine if an LH surge had occurred. Finally, all rats were given 100 mg/kg injection of morphine at 13:30 of proestrus on the sixth cycle.

2.5.2. Experiment no. 2: proestrus administration

Twenty female rats were vaginally smeared for a baseline period of 2 weeks to determine stage of cycle. At 09:00 on proestrus, rats were first given a vehicle injection (cycle 1) and then rested for one cycle. On the third cycle, a clocinnamox injection (10 mg/kg) was given at 09:00 of proestrus. Tail-nick samples for LH were obtained during cycles one and three of proestrus at 11:30 ($n = 7$), at 14:30 ($n = 6$) or 16:30 ($n = 7$). Efficacy of clocinnamox at the earliest time point (2.5 h) was confirmed by assessment of morphine analgesia by tail-withdrawal assay in the group of rats sampled at 11:30. Immediately following their last blood sample, latency to tail-withdrawal following morphine injection (100 mg/kg) was assessed. This study was repeated with an additional group of rats.

2.5.3. Experiment no. 3: diestrus II administration

Twenty-one intact female rats were smeared daily for 2 weeks prior to experimentation and throughout the duration of the experiment. A vehicle injection was administered between 16:00 and 16:30 on the afternoon of diestrus

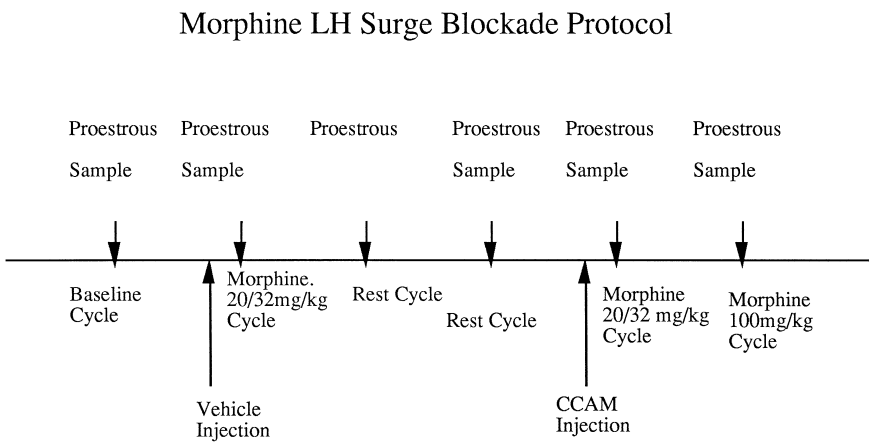

Fig. 1. Diagram of the paradigm demonstrating the blockade of the LH surge by morphine and pharmacological antagonism by clocinnamox. Following several baseline cycles, vehicle was injected on the evening of diestrus II, followed by morphine injection on the afternoon of proestrus, followed by a blood sample for LH at 4:30 in the afternoon (16:30) of proestrus. All rats initially received 20 mg/kg. Those rats which did not demonstrate blockade of the afternoon LH surge received a second morphine injection of 32 mg/kg during proestrus of the following cycle. After successful blockade of the LH surge in all rats and following two rest cycles, rats received clocinnamox on the evening of diestrus II and morphine at the dose which had previously blocked the LH surge, followed by a blood sample for LH at 16:30 of proestrus. On the next cycle, all rats received morphine at 100 mg/kg on the afternoon of proestrus, followed by a blood sample for LH at 16:30 on the afternoon of proestrus.

II. All rats were sampled twice the following day. Blood was collected by tail-nick at 08:30 on the day of proestrus in all rats ($n = 21$) and then at either 12:00 ($n = 7$), 14:30 ($n = 7$), or 16:30 ($n = 7$). Rats were allowed to recover for one complete estrous cycle before administration of 10 mg/kg of clocinnamox, s.c. on diestrus II. Rats then underwent the same sampling paradigm as in the control cycle. Vaginal smears were continued through the entire experimental paradigm and for at least one cycle following the last blood sample to compare pre- and post-clocinnamox treatment cycle lengths. Efficacy of clocinnamox was confirmed by assessment of morphine analgesia (100 mg/kg) by tail-withdrawal assay after the completion of the blood sampling. This same paradigm was repeated with a second group of rats.

3. Results

3.1. Clocinnamox reverses morphine's ability to block the LH surge

Morphine doses of 20 or 32 mg/kg blocked the LH surge in six of six rats (ANOVA, $F(4) = 19.8$, $P < 0.001$; Fig. 2). The second subsequent cycle showed a normal LH surge, suggesting that morphine has no effect 8 days later. Following injection with clocinnamox, morphine no longer produced a blockade of the LH surge. Even a dose of 100 mg/kg of morphine was ineffective in blocking the LH surge. Rats given morphine (20–100 mg/kg) plus clocinnamox treatment showed LH surge levels that were no different than LH surge levels in rats during their control cycle ($P > 0.05$, by post-hoc Scheffe F-test). At the end of the experiment, 6–8 days after clocinnamox injection, the ability of clocinnamox to block morphine's analgesic effect (100 mg/kg) was demonstrated, confirming the efficacy of clocinnamox within the time frame of the experiment (tail-withdrawal latency before morphine, 3.9 ± 0.6 (S.D.) s; after morphine, 5.56 ± 1.0 (S.D.) s; paired t-test, $P > 0.05$).

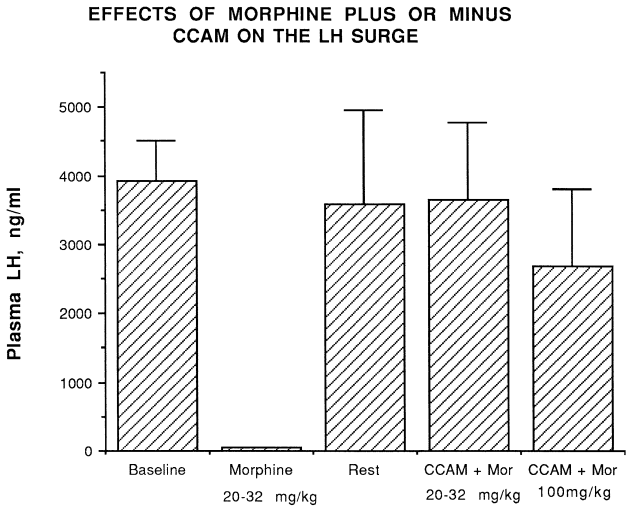

Fig. 2. The blockade of the LH surge by morphine and its reversal by clocinnamox. The first bar shows LH levels during the baseline surge, the second bar following morphine blockade. The cycle after morphine treatment demonstrates a normal LH surge (rest). Clocinnamox reverses the morphine blockade and even blocked the effect of 100 mg/kg of morphine on the LH surge (last bar).

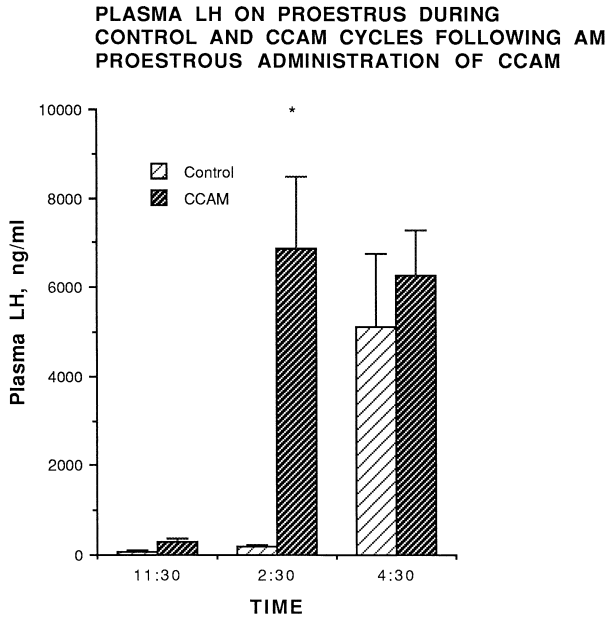

Fig. 3. Effect of clocinnamox administration on the morning of proestrus on the LH surge. Clocinnamox advances the surge by several hours. Maximal plasma LH levels are observed at 2:30 in the afternoon (14:30). (*) Indicates significant differences by post-hoc testing.

3.2. Clocinnamox advances the initiation of the LH surge when given on the morning of proestrus

Administration of clocinnamox at 09:00 on proestrus led to a significant increase in plasma LH by 11:30 (Fig. 3). The increase in LH seen at 11:30 was indeed the beginning of the LH surge, as the levels in clocinnamox rats remained elevated at 14:30. In fact, LH levels were 10-fold higher in clocinnamox-treated rats than controls at 14:30. The LH levels at 14:30 in clocinnamox-treated rats were similar to peak LH surge levels seen at 16:30 on proestrus before clocinnamox treatment. Two-way ANOVA revealed a significant effect of time ($F(2) = 61.46$, $P < 0.0001$), a significant effect of clocinnamox ($F(1) = 53.7$, $P < 0.0001$), and a significant interaction ($F(2) = 13.6$, $P < 0.01$). Following clocinnamox treatment, plasma LH remained at peak levels between 14:30 and 16:30. A repeat experiment showed similar significant effects.

Rats treated with clocinnamox on the morning of proestrus showed a complete blockade of morphine analgesia when evaluated 2.5–3 h after clocinnamox treatment. There was no significant difference in latency to tail-

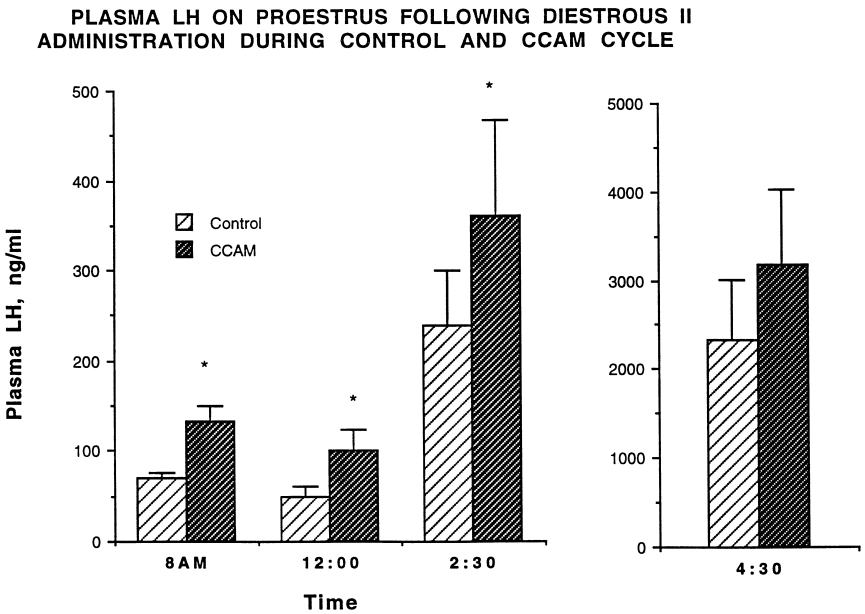

Fig. 4. The effects of clocinnamox administration on the evening of diestrus II on the LH surge. Unlike proestrus administration, clocinnamox administration did not advance the surge but rather increased LH levels throughout the day of proestrus, without altering the timing of the LH surge. (*) Indicates significant differences by post-hoc testing.

withdrawal in rats prior to or after administration of 100 mg/kg of morphine (paired *t*-test, $P > 0.05$), indicating that clocinnamox was effective by 2.5 h after administration. There was no apparent change in cycle length with proestrus AM administration of clocinnamox. Before clocinnamox treatment, the average cycle length was 4.09 $\pm$ 0.03 days. After clocinnamox treatment, it was 4.2 $\pm$ 0.08 days. These values did not differ significantly (paired *t*-test, $P > 0.05$).

3.3. Clocinnamox does not alter the circadian time of the LH surge when given on the afternoon of diestrus II

Given that clocinnamox was able to advance the surge when given on proestrus, the critical question of whether removal of opioid tone on diestrus II would advance the surge was addressed. In contrast to the effect seen with proestrus administration of clocinnamox, diestrus II administration of clocinnamox caused an elevation in plasma LH levels across the day of proestrus with normal timing of the LH surge (Fig. 4, two-factor ANOVA, effect of clocinnamox treatment, $P < 0.01$, $F(1) = 10.4$; effect of time of day, $P < 0.01$, $F(3) = 46.9$; and no interaction, $P > 0.05$, $F(3) = 0.458$). At each time point prior to the initiation of the LH surge, there was a 30%–50% increase in plasma LH levels following clocinnamox treatment. Except for the 16:30 surge data, all the LH levels were significantly different from control cycle when compared by post-hoc testing ($P < 0.05$). In a repeat experiment, a similar pattern was seen. In neither experiment, nor in a combined analysis of data from both experiments did LH levels during the LH surge differ significantly between control and clocinnamox days (one-way ANOVA, $P > 0.05$). There was no indication that the timing of the LH surge was altered by clocinnamox treatment. In fact, in the initial study, two rats showed surge levels of LH by 14:30 in both their control and clocinnamox-treated cycles. Although this is somewhat earlier than most rats in the study, they did not begin their surge earlier in the clocinnamox cycle. There was no apparent change in cycle length with diestrus II PM administration of clocinnamox. Before clocinnamox treatment, the average cycle length was 4.1 $\pm$ 0.05 days. After clocinnamox treatment, it was 4.3 $\pm$ 0.08 days. These values did not differ significantly, (paired *t*-test, $P > 0.05$).

4. Discussion

The initial experiments demonstrated that clocinnamox is an effective tool for the study of the regulation of LH secretion in female rats. Based upon its ability to antagonize morphine's analgesia for periods greater than 1 week, and its reduction in μ-opioid receptor binding as determined by receptor binding assays when administered in vivo (Paronis and Woods, 1997), clocinnamox is a long-acting μ-opioid receptor antagonist. Furthermore, clocinnamox's long duration of opioid blockade enabled us to vary the phase of the estrous cycle where endogenous opioid tone was removed and to maintain the blockade for more than one estrous cycle.

The data presented here indicate that administration of clocinnamox on the morning of proestrus was able to advance the LH surge while administration of clocinnamox in the evening of diestrus II, 24 h in advance of the surge,

did not alter the timing of the surge but increased plasma LH levels throughout the day of proestrus in comparison to the sham cycle. These studies support a role for opioid tone in the regulation of LH secretion and confirm earlier findings that blockade of opioid tone on the morning of proestrus can advance the LH surge (Allen et al., 1988; Allen and Kalra, 1986). Kalra et al. were similarly unable to advance the LH surge when they gave naloxone treatment very early in the morning of proestrus (personal communication). The inability to advance the surge by more than a few hours may reflect the underlying limitations on other aspects of the LH surge mechanism. This includes the lack of readiness of the pituitary either in terms of LH stores or GnRH responsiveness, the possibility that hypothalamic GnRH neurons are incapable of initiating a GnRH surge at an earlier time, or that GnRH neurons and LH cells do not have sufficient peptide stores to respond to removal of opioid tone.

When the timing of complete opioid blockade is compared to the actual time of the surge, analgesia is completely blocked by 2.5 h and maximal reduction in opioid receptor binding occurs by 2 h (Paronis and Woods, 1997). The plasma LH levels in clocinnamox-treated rats in the two proestrus administration experiments are elevated at this time (11:30), but in no experiment is it to surge levels. Although an earlier LH surge occurred in the proestrus administration experiment, this was still only 2 h in advance of the normal surge. Furthermore, the discrepancy between diestrus II and proestrus administration suggest that a specific steroid milieu is necessary when opioid tone is removed in order to advance the LH surge. This is further supported by studies of Lustig et al. (1988) who found that implantation of Alzet minipumps containing naloxone on diestrus II did not advance the surge but led to an increase in serum LH levels from 13:00–19:00, while rats treated acutely with nalmefene on proestrus showed advanced LH surges. Alternatively, 'tolerance' may develop to the ability of opioid antagonists to regulate LH secretion. Tolerance to the ability of naloxone pellets to increase serum LH in ovariectomized rats has been reported (Gabriel and Simpkins, 1983). In that study, the 'tolerance' did not appear to be due to lack of estradiol, since acute naloxone challenge in ovariectomized rats retained its ability to raise LH levels. Furthermore, naloxone pellets were able to prevent morphine-induced decreases in LH secretion. However, contrary to the data in the current study, they found no effect of naloxone pellets on baseline LH secretion in ovariectomized rats replaced with estradiol. The increase in LH secretion seen throughout the day of proestrus observed in clocinnamox-treated rats suggest that clocinnamox is still affecting the opioid circuits modulating LH secretion, but that it no longer affects the timing of the surge. Thus, the most consistent explanation is that a specific steroid milieu is necessary at the time of opioid withdrawal in order to advance the surge. This suggests a narrow window of opioid sensitivity in the LH surge mechanism, since antagonists can only advance the surge when given on the morning and afternoon of proestrus, and morphine can only block the surge if administered before 14:30.

Chronic clocinnamox treatment did not alter cycle length in any of the experiments described above, indicating that changes in opioid tone at the μ-opioid receptor are not necessary for the transition between any of the phases of the cycle. This agrees with previous studies using naloxone pellets (Pfeiffer et al., 1984). These studies with clocinnamox, a μ-specific opioid receptor antagonist, show findings similar to that found with naloxone infusion or pellets, suggesting that on the day of proestrus, μ-opioid receptors are the critical receptors involved in opioid modulation of basal LH secretion and of the LH surge mechanism. The findings of μ-opioid receptor specificity lends further support to the importance of β-endorphin as the primary opioid peptide system involved in the regulation of GnRH secretion. This does not exclude the possibility that δ-opioid receptors, for which β-endorphin also shows great affinity, may play an adjunctive role. In fact, the report by Wiesner et al. (1985) that ICI 54, 129 could reverse the effect of infused β-endorphin, suggest that δ-opioid receptors may also participate in the response to β-endorphin. Interestingly ICI 154, 129 had no effect on baseline LH secretion, emphasizing that different opioid receptors may mediate baseline vs. stress vs. surge modes of LH secretion. These experimental findings with long-term μ-opioid receptor antagonism with clocinnamox contradict the hypothesis supported by Kalra (1993) and Piva et al. (1985) that there is opioid tone at all times during the estrous cycle, except on the day of proestrus. Rather, our data support the hypothesis that there is opioid tone on proestrus before the onset of the LH surge. These data do not conclusively answer whether there is opioid tone during the LH surge, but suggest that removal of opioid tone is not the trigger for the initiation of the LH surge.

5. Conclusion

This observation, combined with the findings that a very high dose of morphine is needed to block an LH surge and that this blockade only occurs when the morphine is given at a precise time of day on proestrus, leads us to the hypothesize that there is an opioid-sensitive window on proestrus. This window appears to include the morning and early afternoon of proestrus. Opioid antagonists such as naloxone or clocinnamox given earlier than 09:00 and maintained throughout proestrus do not change the timing of the LH surge. Opioid agonists such as morphine given after 14:00 will not inhibit the LH surge. Furthermore, this effect is mediated entirely through the μ-opioid receptor, since clocinnamox can replicate the advanced LH surge found with naloxone administration on the morning of proestrus.

Acknowledgements

The authors would like to acknowledge the support of MH 45232 (EY) and MH 00427 (EY) and DA 00254 (JHW) and the P30 HD 18258 Laboratory Animal Core and Assay and Reagents Core.

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