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GONADAL STEROID MODULATION OF BRAIN OPIOID SYSTEMS

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Summary—It is becoming increasingly clear that the effects of the opioids and their synthetic analogs on anterior pituitary function largely depend on the steroid milieu present in the animal at time of drug administration. However, it is still unclear whether gonadal steroids regulate the opioid-modulated mechanisms by affecting the number of opiate receptors in the brain. To further investigate these issues, the effects of opiate agonists and antagonists on LH, FSH and prolactin (Prl) secretion have been studied in: (a) normal and castrated male rats, and (b) normally cycling female rats. The binding characteristics of the brain subclass of mu opiate receptors have been analyzed in the same group of experimental animals; this type of receptors seems to be particularly involved in the control of gonadotropin and Prl release.

When injected intraventricularly into normal male rats, morphine (200 μg/rat) induced in a significant elevation of serum LH levels at 10 and 20 min. In long-term castrated animals the administration of the drug significantly reduced LH secretion at 40 and 60 min after the injection, the inhibition lasted up to 180 min. Morphine, when given intraventricularly to normal males, induced a conspicuous and significant elevation of serum Prl levels at 10, 20, 40 and 60 min after treatment. However, when the drug was administered to castrated rats, it did not significantly affect Prl release at any time interval considered. Morphine intraventricular injections did not modify serum FSH levels either in normal or in castrated male rats. The concentration of mu opiate receptors was found to be similar when measured in the whole brain of normal and orchidectomized rats.

In adult cycling female rats, s.c. injections of naloxone (2.5 mg/kg) stimulated LH release in every phase of the estrous cycle; the magnitude of the responses was highly variable, being particularly elevated at 16.00 h of the day of proestrous and at 10.00, 12.00 and 14.00 h of the day of estrous. Conversely, LH response to naloxone was totally obliterated at 18.00 and 20.00 h of the day of proestrous, when the preovulatory LH surge was found to occur. The concentration of brain opiate receptors of the mu type showed significant variations during the different phases of the estrous cycle, with higher levels at 12.00 h of the day of proestrous and at 18.00 h of the day of estrous.

These results suggest that (1) gonadal steroids play a major role in directing the effects of the exogenous opiate agonists and antangonists on gonadotropin and Prl secretion both in male and in female rats; (2) in male rats, gonadal steroids do not develop their central effects by modulating the brain mu population of opiate receptors; (3) in female rats, the concentration of brain mu opiate receptors changes during the different phases of the estrous cycle, possibly as a consequence of the fluctuating levels of gonadal steroids.

INTRODUCTION

The opioid peptides are highly localized in the hypothalamus and in other areas of the brain which are known to participate in neuroendocrine central processes [1]. Specific receptors for the opioids, especially of the mu, kappa and delta type, have also been demonstrated to be present in several CNS areas. These observations suggest that brain opioid peptides might participate in the control of anterior pituitary function. It is now generally accepted that the opioids exert a stimulatory effect on prolactin secretion [2, 3]. Opioid peptides also participate in the control of gonadotropin release. The majority of the data available suggest that they normally inhibit gonadotropin secretion [2, 3]; however, recent reports underline the possibility that, in particular experimental conditions, these peptides and their synthetic analogs might stimulate rather than inhibit LH release [4–13].

Recently, it has been shown that the effects of the opioids and of their analogs on gonadotropin secretion largely depend on the steroid milieu present in the animal at time of drug administration. Naloxone has been reported to increase serum LH levels in intact adult male rats [14–17] and in orchidectomized-androgen-treated animals [16–19] but not in long-term orchidectomized untreated rats [17]. Bhanot and Wilkinson[20] have shown that the enkephalin analog FK 33824 decreases serum LH levels in male and in female rats 2 days but not 7 days after gonadectomy, and that the ability of FK 33824 to inhibit LH release is restored, in long-term gonadectomized animals, by the administration of androgens or estrogens. Blank *et al.*[14] have reported that naloxone does not affect LH secretion in adult female rats; other authors [21–25], however, have reported that the stimulatory effect of naloxone on LH release occurs also in adult females.

The experiments here to be described have been performed in order (1) to gain additional information on the effects exerted by gonadal steroids on the responsiveness of the hypothalamo-pituitary axis to the administration of opiates, and (2) to verify whether gonadal steroids exert their effects on opioid-modulated mechanisms by affecting the subpopulation of mu brain opiate receptors; this type

of receptors seems to be particularly involved in the control of gonadotropin and prolactin secretion [26, 27]. To this purpose, the effects of opiate agonists and antagonists on gonadotropin and prolactin secretion have been studied in the following groups of experimental animals: (a) normal and castrated male rats, and (b) normally cycling adult female rats. In the same groups of experimental animals, the binding characteristics (number of receptors, B_{max}, and constant of affinity, K_a) of brain mu opiate receptors have also been analyzed.

RESULTS AND DISCUSSION

Experiments in normal and castrated male rats

Effects of morphine on gonadotropin and prolactin secretion. In order to evaluate the role exerted, in male animals, by the steroid milieu on the response of the two gonadotropins (LH and FSH) and of prolactin to the administration of the opiates, morphine (an opioid agonist) has been injected into normal or long-term castrated male rats. The drug has been administered into the cerebrospinal fluid via intraventricular injections; serum levels of LH, FSH and prolactin have been subsequently evaluated at different time intervals, by using specific radioimmunoassays.

Figure 1 (upper panel) shows that morphine, when injected intraventricularly into normal adult male rats in the dose of 200 μg/rat induces a conspicuous and significant elevation of serum LH levels at 10 and 20 min. Figure 1 (lower panel) also shows the results obtained following the intraventricular administration of the same dose of morphine to adult castrated male rats. In castrated animals, the injection of morphine was not followed by any significant modification of serum LH levels 10 and 20 min after treatment. However, morphine administration brought about a conspicuous and significant decrease of serum LH levels at 40 and 60 min. The inhibitory effect of morphine on LH secretion lasted up to 180 min after injection (data not shown).

FSH secretion was not affected by the injections of morphine either in normal or in castrated males at any time considered (data not shown).

Figure 2 shows the effects on serum prolactin concentrations of 200 μg/rat morphine administered intraventricularly to normal (upper panel) and castrated (lower panel) adult male rats.

In normal male animals morphine significantly enhanced prolactin release at all the times considered; on the other hand, the drug was totally unable to increase prolactin release in adult castrated male rats at any time after injection.

The results of the present experiments show, first of all, that morphine may participate in a stimulatory way in the control of LH release. This observation, which appears to contrast with several literature data suggesting that brain opioids exert a tonic inhibitory role in the control of LH secretion [3, 15, 18], finds support in a series of recent studies. Opioid peptides and synthetic opiate analogs have indeed been shown to stimulate LH release in different experimental conditions. In particular, Takahara *et al.*[5]

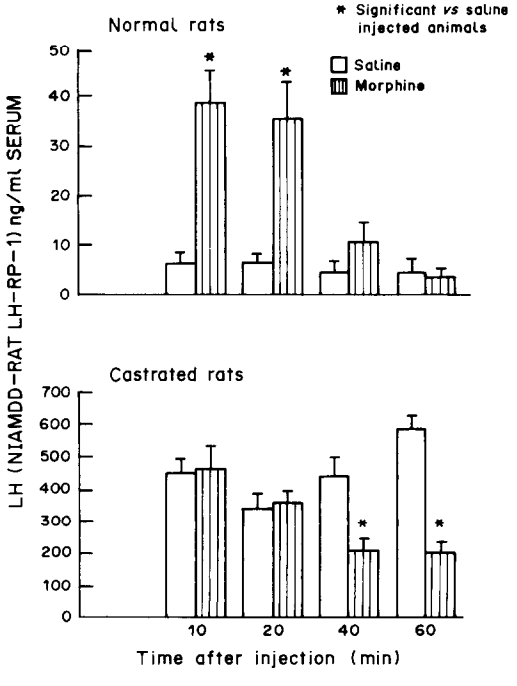

Fig. 1. Effects of intraventricular injections of morphine (200 μg/rat) on serum LH levels of adult normal and castrated (4 weeks) male rats.

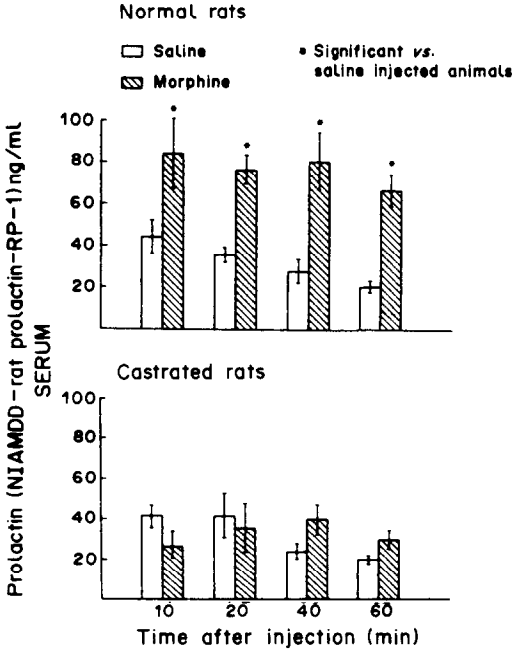

Fig. 2. Effects of intraventricular injections of morphine (200 μg/rat) on serum prolactin levels of adult normal and castrated (4 weeks) male rats.

have found that intraventricular injections of β-endorphin in normal male rats increase serum LH levels in a dose-dependent fashion. Motta and Martini[9] have found that met-enkephalin and one of its analogs (D-ala^2-met-enkephalinamide) increase serum LH levels when given intraventricularly to long-term castrated female rats. Leu-enkephalin and the met-enkephalin analogs FK 33824 and D-ala^2-met-enkephalinamide have been found to strongly stimulate LH release in ovariectomized female rats following intraventricular injection [13, 28]. It is interesting to underline that, in agreement with the data here presented, all these authors reported a stimulatory effect of the opiate agonists on LH release after intraventricular injections of the various drugs.

The finding that, in given conditions, morphine and several opioids or opiate analogs may stimulate LH release suggests the presence in the brain of some opioid-modulated system(s) which exert(s) stimulatory influences on LH secretion. This activatory mechanism is probably complementary to the inhibiting one described in the literature (see 2, 3 for references).

It appears possible that morphine and opiate analogs, when injected intraventricularly, might reach the stimulatory system(s) more easily than the inhibitory one(s).

The data here presented have clearly indicated that the stimulatory effect of intraventricular injections of morphine on LH release disappears following castration. Actually, morphine was found to significantly decrease serum LH levels from 40 to 180 min following intraventricular injection into long-term castrated male rats.

Therefore, these data suggest that the presence or absence of endogenous androgens may be crucial in modulating the effects of the opioids on LH secretion.

The results of the present study also show that morphine, when given intraventricularly to normal adult male rats, is able to increase serum prolactin levels. These data are in agreement with results obtained by previous authors, following systemic [2, 3] or intraventricular injection of morphine, endogenous opioid peptides or their synthetic analogs [13, 29–36]. On the contrary, experiments performed in long-term castrated rats clearly show that the intraventricular injection of morphine is totally unable to increase serum prolactin concentrations following orchidectomy. There is only little information in the literature supporting the view that the absence of testicular steroids might alter the prolactin response to the administration of morphine or other opioids. Goldberg *et al.*[37] have shown that the intraventricular administration of 500 μg met-enkephalin to normal adult male rats causes a significant rise in serum prolactin concentrations and that the same treatment results in a much lower release of prolactin in orchidectomized rats. Kato *et al.*[34] have found that β-endorphin induces an increase of plasma prolactin in castrated male rats which is almost 10 times lower than that observed in normal animals [31]. Moreover, Forman *et al.*[38] have reported that the stimulatory effect of morphine on prolactin release is decreased in aged male rats, which are known to have serum testosterone levels lower than those of younger animals. Foresta *et al.*[39] have also reported that the enkephalin analog FK 33824 significantly increases prolactin levels in normal men, but is much less effective in castrated subjects.

In general, the data here reported show that, in male rats, the effects of intraventricular injections of morphine on LH and prolactin secretion largely depend on the presence or absence of testosterone.

It is believed at present that the opioids may modify anterior pituitary function acting on the nervous system through the activation of specific brain receptors. In particular, the mu receptors seem to mediate the opiod effects on LH release, while the activation of the mu and kappa receptors seems to be involved in the regulation of prolactin secretion [27, 40, 41]. It is then possible to suggest that the different effects exerted by morphine on LH and prolactin release in normal and in castrated male rats might be due to an alteration, induced by the removal of gonadal steroids, of the number or of the binding characteristics of brain mu and/or kappa receptors. This hypothesis has been tested in the following experiments.

Brain mu opiate receptors in normal and castrated male rats. In order to test this hypothesis, the concentration (B_{max}) and the constant of affinity (K_a) of mu receptors have been analyzed, by means of a specific radioreceptor assay, in the whole brain of normal and long-term (4 weeks) castrated male rats. [^{3}H]DHM (dihydromorphine) was used in the radioreceptor assay as a specific binder for the mu class of opiate receptors. The receptor assay has been performed as described elsewhere [42].

Table 1 shows that gonadectomy does not modify the [^{3}H]DHM opiate receptor affinity. Since this parameter was not modified by orchidectomy, the concentration of mu binding sites was measured in individual brain membrane preparations in the two experimental groups. As shown in Table 1, the concentration of opiate receptors binding [^{3}H]DHM was not significantly affected by castration.

Table 1. Effects of castration on [^{3}H]DHM binding in the whole brain of male rats

	Constant of affinity (K_a) (M^{-1})	Binding capacity (B_{max}) (fmol/mg protein)
Normal males	1.47×10^9	(11)* 122.69 ± 2.05
Castrated males	1.56×10^9	(10) 122.04 ± 10.09

*Number of animals is shown in parentheses.

These results show that, in male rats, testicular steroids do not modulate the binding parameters of brain mu opiate receptors. This observation does not agree with that of Hahn and Fishman [43, 44], who reported that castration, performed 3 weeks before, results in a significant increase of the B_{max} of [^{3}H]naltrexone and [^{3}H]naloxone binding sites in the brain of male rats. The reason for the discrepancy between the present results and those of Hahn and Fishman [43, 44] may be found in the differences between the procedures adopted for the receptor assay. In particular, it must be underlined that the choice of the ligand for the binding assay appears to be of great importance for the analysis of brain opiate receptors. For their experiments, Hahn and Fishman[43, 44] utilized [^{3}H]naltrexone and [^{3}H]naloxone, which have been demonstrated to be ubiquitous ligands for all types of opiate receptors; therefore, the use of these drugs does not allow a specific analysis of the effects of gonadal steroids on single subclasses of opiate receptors. In the present study, [^{3}H]DHM was used as the ligand since this compound is believed to bind selectively to mu opiate receptors.

The results here presented are, however, in agreement with those reported by other authors [45–47]. In 1981, Wilkinson *et al.*[45] demonstrated that, in adult male rats, the number of brain opiate receptors is not significantly modified by orchidectomy performed 3 weeks before. Similar results were obtained also in castrated animals treated daily with testosterone propionate for 7 days. The analysis of the number of mu opiate binding sites were performed by these authors [45] using different types of ligands believed to be "specific" for the different subpopulations of opiate receptors ([^{3}H]DHM, a mu-receptor ligand; [^{3}H]D-alanine-D-leucine enkephalin, DADLE, a delta-receptor ligand with a minor mu component, and [^{3}H]naloxone, an ubiquitous ligand). Later, Diez and Roberts[46] also reported that orchidectomy performed 3 weeks before the experiment, does not influence the concentration of [^{3}H]naloxone binding sites in the whole brain of male rats and mice. Moreover, these authors [46] have found that the administration of testosterone (either subcutaneously or via silastic capsules) to intact male mice does not alter the brain concentration of opiate receptors. Finally, Cicero *et al.*[47] found that also long-term castration (1–3 months) fails to produce any significant change in the K_d (dissociation constant) or in the B_{max} values of mu, delta and naloxone binding sites in the whole brain or in the hypothalamus of male rats.

In conclusion, the data here discussed show that orchidectomy does not modify the binding parameters (K_a and B_{max}) of brain mu opiate receptors in male rats. These data suggest that, in male rats, gonadal steroids do not modulate the responsiveness of the hypothalamo-pituitary axis to the administration of the opiates by affecting the population of brain mu opiate receptors.

Experiments in female rats

Effects of naloxone on gonadotropin secretion. These experiments have been performed in order to analyze whether the changes in the levels of estrogens and progesterone, which spontaneously occur during the different phases of the estrous cycle of the female rat [48], might influence the effects of naloxone on LH and FSH secretion. To this purpose, naloxone has been injected subcutaneously at different times of the day during the various phases of the estrous cycle; naloxone-treated animals and the respective saline-treated controls have been sacrificed 20 min after injection. Blood samples were collected for serum LH and FSH levels determinations.

It is evident from Fig. 3 that, in the saline-treated animals, serum LH is low during the days of diestrus

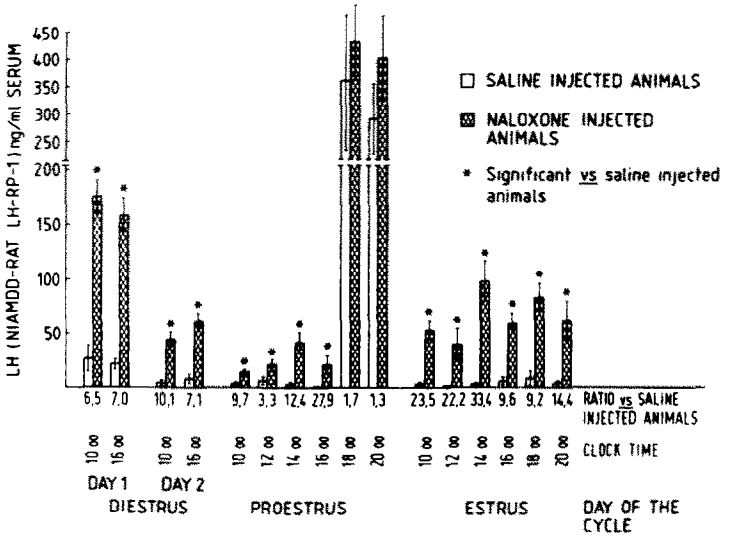

Fig. 3. Effects of s.c. injections of naloxone (2.5 mg/kg) on serum LH levels of adult cycling female rats treated at different hours of the day throughout the estrous cycle. Animals were killed 20 min after naloxone administration. The hours in the graph refer to the actual time of death.

1 and 2 and during the day of estrous; the expected LH surge of the day of proestrous begins after 16.00 h, reaches a maximum at 18.00 h and starts declining at 20.00 h. The s.c. administration of naloxone is followed by an increase of serum LH levels on every day of the estrous cycle (Fig. 3). However, the magnitude of the effects exerted by naloxone on LH release was different on the various days; this clearly appears from Fig. 3, in which the ratios of LH values in naloxone-treated rats vs LH values in saline-injected animals have been reported.

The administration of naloxone brings about a moderate increase of serum LH levels during the days of diestrus 1 and 2; during these these two days, the effects exerted by naloxone on LH secretion are quantitatively similar at 10.00 and at 16.00 h. During the day of proestrous naloxone exerts a stimulatory effect on LH release quantitatively similar to that observed during the days of diestrous 1 and 2 at 10.00 and at 14.00 h; at 12.00 h the effect of naloxone appears to be lower. On the contrary, a major increase of the stimulatory effect of naloxone on LH release is observed at 16.00 h; the increase in serum LH levels induced by naloxone at 16.00 h is significantly higher than those found at 10.00, 12.00 and 14.00 h. After 16.00 h of the day of proestrus the effect of naloxone on LH secretion disappears; the values of serum LH in the naloxone-treated animals recorded at 18.00 and 20.00 h are not significantly different from those found in the corresponding saline-treated controls. During the day of estrous a very significant LH response to naloxone occurred at 10.00, 12.00 and 14.00 h; at these time intervals, the increase in serum LH levels induced by the treatment with the drug are similar to that observed at 16.00 h of the day of proestrous. At later times, the effects of naloxone on LH secretion appear to diminish; the increases observed at 16.00, 18.00 and 20.00 h are similar to those found during the day of diestrous 2.

Throughout the experiments here reported there was no effect of the administration of naloxone on FSH release. In no instance were the values of serum FSH recorded after naloxone administration significantly different from those of the corresponding controls (data not shown).

The present data show, first of all, that the s.c. administration of naloxone is effective in inducing significant increases in serum LH levels during every day of the estrous cycle. This finding suggests that the endogenous opioids may exert an inhibitory effect on LH release also in normally cycling adult female rats. This observation, which is in contrast with the results of Blank *et al.*[14], finds support in data published by Gabriel *et al.*[21, 24], who also found naloxone to be able to stimulate LH release in adult female rats during the various phases of the estrous cycle. The data of the present study also clearly indicate that the stimulatory effect naloxone exerts on LH release exhibits conspicuous and significant variations throughout the four days of the estrous cycle. In particular, these results confirmed [21] that the respones to naloxone are quantitatively similar in the second day of diestrous and on the day of proestrous (at 10.00, 12.00 and 14.00 h), prior to the preovulatory LH surge. Moreover, they also show that naloxone becomes much more effective in facilitating LH release at 16.00 h of the day of proestrous, i.e. just before the initiation of the spontaneous proestrous LH surge. It is believed that this increased sensitivity to naloxone does not underline a change in the opiatergic tone controlling LHRH secretion, but only reflects the increased responsiveness of the anterior pituitary to endogenous LHRH induced by the elevated estrogenic secretion present at this time [49, 50].

The increased sensitivity to naloxone of the mechanisms controlling LH secretion during the day of proestrous appears to be of short duration: the drug totally loses its ability to stimulate LH secretion during the preovulatory LH surge (at 18.00 and 20.00 h). Similar results have been recently reported by Petraglia *et al.*[25]. These authors have shown that naloxone is able to stimulate LH secretion during the different phases of the estrous cycle, but not in the afternoon of proestrous. Moreover, Gabriel *et al.*[21, 24] have observed that the ability of naloxone to stimulate LH release is reduced during the LH surge induced by estradiol benzoate-progesterone (EBP) treatment in overiectomized rats. Gabriel *et al.*[24] have also shown that this decline in the secretory response to naloxone during EBP-induced LH surge is not due to changes in the response of the pituitary to LHRH. Actually, when these animals were treated with exogenous LHRH, they still presented a significant increase in LH secretion. The data of the present experiments, together with those of Petraglia *et al.*[25] and Gabriel *et al.*[21, 24], clearly indicate that during the period of the proestrous LH hyper-secretion, the influence of endogenous opioids on LH release is totally blunted. It still remains to verify whether this change of the opiatergic tone controlling LH secretion is a primary phenomenon, or whether it is secondary to the effects of ovarian steroids [progesterone or other progestagens (e.g. 20 α-OH-progesterone, etc.)], whose secretion significantly increases during the late afternoon of the day of proestrous [51].

Finally, the present study has clearly demonstrated that the sensitivity of LH to naloxone reappears during the day of estrous; this increased sensitivity may be due to a change occurring in the opiatergic tone controlling LHRH secretion. It has actually been reported that naloxone may facilitate LH release via a hypothalamic mechanism which results in a stimulation of LHRH secretion [52, 53]. Again, this change in the opiatergic tone controlling LH release may be a primary phenomenon occurring at the central level. However, one cannot exclude that it might be secondary to the effects of ovarian steroids secreted during the day of proestrous; it is indeed possible that the endogenous

secretion of estrogens adn/or progesterone acts at the central level affecting the brain opioid peptide systems.

Brain mu opiate receptors in female rats. The data discussed in the previous section of this paper indicate that the activity of the central opiatergic tone controlling LH secretion fluctuates in female rats throughout the different phases of the estrous cycle; this fact might be mediated by changes in the binding characteristics of the brain mu opiate receptors. To test this hypothesis, the affinity and the number of brain receptors of the mu type have been analyzed in the whole brain of female rats during the different phases of the estrous cycle. The results of the present experiment are summarized in Fig. 4 (upper panel). It is clear that the number of brain receptors of the mu type shows significant variations during the different phases of the estrous cycle. Their number is rather low at 10.00 and 16.00 h of the second day of diestrous and at 10.00 h of the day of proestrous. A significant increase of the number of brain mu receptors is observed at 12.00 h of this day. After this time, there is a progressive decline of the number of the mu receptors which, at 18.00 h, returns to the level found at 10.00 h. The number of brain mu receptors remains then low and rather constant up to 16.00 h of the day of estrous; a significant increase occurs again at 18.00 h of this day. This increase is then followed by a decrease which, during the first day of diestrous, gradually brings the number of receptors to the low levels observed during the second day of diestrous.

Figure 4 (lower panel) shows serum LH levels measured in the animals included in this study. It can be seen that the LH surge begins at 14.00 h of the day of proestrous and that it reaches its maximal

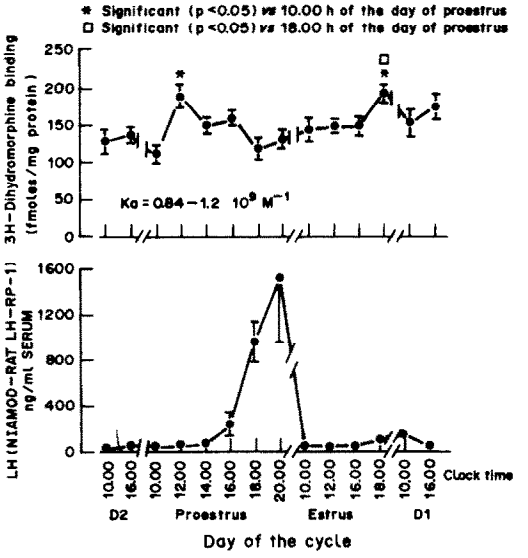

Fig. 4. Concentration of brain [^{3}H]DHM binding sites during the various phases of the estrous cycles of female rats.

levels at 18.00 h. This in agreement with the previous data of this and other laboratories [48–51, 54–57].

The present data indicate that the number of opiate receptors binding dihydromorphine shows conspicuous changes in the whole brain of the female rat during the various phases of the estrous cycle, with an increase in concentration at 12.00 h of the day of proestrous and at 18.00 h of the day of estrous. At present, two possibilities may be considered in order to explain these estrous-linked modifications of the number of brain mu opiate receptors. First of all, they may represent a primary phenomenon occurring at central level. Second, these modifications may be induced by changes of the steroid "milieu" occurring during the different phases of the estrous cycle. According to this second hypothesis, it might be speculated that the number of brain mu receptors increases during the day of proestrous under the influence of estrogens secreted during this phase of the ovulatory cycle [48–50] and that their decline during the late phases of the day of proestrous and during estrous are the consequence of the concomitant effects of estrogens and progesterone secreted during these phases [48–51]. However, the increase of the number of mu opiate receptors occurring on the evening of the day of estrous appears difficult to explain on the basis of the changes of the steroid "milieu" since in this phase both estrogens and progesterone remain constant. Recently, Wilkinson *et al.*[58, 59] have demonstrated a significant increase in [^{3}H]naloxone binding in the anterior hypothalamus of ovariectomized female rats following the chronic implantation (12 weeks) of silastic capsules filled with estradiol or the s.c. administration of estradiol valerate. However, in their papers, Wilkinson *et al.*[58, 59] did not specify whether the increase in the binding was due to a change of the B_{max} or of the K_a of the ligand for the opiate receptors.

It appears difficult to strictly compare the results here obtained with those previously reported on the effects of naloxone on LH release during the different phases of the estrous cycle. Actually, naloxone has been shown to induce only a moderate LH response at 12.00 h of the day of proestrous and at 18.00 h of the day of estrous, when the number of brain mu opiate receptors is elevated. The apparent disrepancy between the two groups of results may be due to several reasons. First of all, in the present study the concentration of opiate receptors has been evaluated in the whole brain; possibly more accurate information could come from an analysis of the number of the receptors in the hypothalamus and in other structures directly involved in the control of gonadotropin secretion. This study is at present in progress in this laboratory. Second, the possibility cannot be excluded that opiate receptors other than the mu subclass (delta and kappa) might participate in such a control.

CONCLUSIONS

In summary, the experiments discussed in the present paper have shown that

(1) in male rats gonadal steroids seem to play a major role in directing the activity of exogenous opiates;

(2) in male rats, gonadal steroids do not seem to develop their central effects by affecting brain mu opiate receptors;

(3) in female rats, the tonic inhibitory tone exerted by brain opioids on LH secretion exhibits conspicuous and significant variations throughout the estrous cycle, possibly as a consequence of the fluctuating levels of gonadal steroids;

(4) an interaction between gonadal steroids and the brain opioid system, in regularly cycling female rats seem to be confirmed by the changes in the concentration of brain mu opiate receptors observed during the different phases of the estrous cycle.

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