J. Steroid Biochem. Molec. Biol. Vol. 40, No. 1–3, pp. 113–121, 1991
Printed in Great Britain. 

0960-0760/91 $3.00 + 0.00


# MODULATION OF THE BINDING CHARACTERISTICS OF HYPOTHALAMIC MU OPIOID RECEPTORS IN RATS BY GONADAL STEROIDS

ROBERTO MAGGI,* PATRIZIA LIMONTA, DONATELLA DONDI and FLAVIO PIVA
Department of Endocrinology, University of Milano, Via G. Balzaretti, 9 20133 Milano, Italy

**Summary**—Recent evidence suggests that the effects of the opioids on gonadotropin release may depend on the endocrine status existing in the experimental animal. In the brain, the effects of the opioids are exerted through the interaction with different classes of opioid receptors (mu, delta, kappa, etc.). Among these, the mu receptors appear to be particularly relevant to the control of gonadotropin secretion. Different groups of experiments have been performed in the rat in order to analyze whether changes of circulating levels of sex steroids may have an impact on the binding characteristics of hypothalamic mu opioid receptors, as evaluated by a receptor binding assay performed on plasma membrane preparations, using [$^3$H]dihydromorphine as a mu ligand. In a first series of experiments, it has been observed that the ontogenesis of hypothalamic mu opioid receptors is different in male and in female rats: the concentration of mu sites, similar in animals of the two sexes at 16 days of age, increases in females, but not in males, between day 16 and day 26 of life. This sexual difference persists in 60-day old animals, when the brain is fully mature. It has also been observed that the pattern of maturation of hypothalamic mu receptors can be reversed by neonatal castration of males and by neonatal testosterone treatment of females. In a second series of experiments, it has been shown that in the hypothalamus of regularly cycling female rats the concentration of mu receptors varies during the different phases of the estrous cycle. In particular, a rather high density of mu sites during diestrus day 2 and the morning of the day of proestrus was found; this is followed by a progressive decline during the afternoon of the day of proestrus and the day of estrus, with a minimum value of the concentration of mu receptors being recorded in the first day of diestrus. These fluctuations seem to be linked to the physiological changes of serum levels of ovarian steroids: in fact, in a third series of experiments, it has been found that the positive feedback effect on LH release, exerted by the treatment of ovariectomized female rats with estrogens plus progesterone, is accompanied by a significant decrease of the concentration of hypothalamic mu opioid receptors; treatments with estrogens alone, able to induce a negative feedback effect on LH secretion, are not associated with modifications of hypothalamic mu receptors. These data seem to indicate that hypothalamic mu receptors may be involved in the positive but not in the negative feedback control of LH secretion. Finally, it has been observed that during pregnancy there is a significant increase of the concentration of hypothalamic mu opioid receptors followed by a return towards control levels during the postpartum period. On the other hand, the simulation of the levels of sex steroids present during the first half of pregnancy, obtained through a long-term treatment of ovariectomized animals with estrogens plus progesterone, was not accompanied by any modification of the concentration of hypothalamic mu receptors. On the basis of these last results, the authors would then be inclined to suggest that, during pregnancy, the concentration of hypothalamic mu opioid receptors is not linked to endocrine phenomena, but may be related to the stressful conditions represented by pregnancy itself and by the oncoming delivery. The present findings strongly suggest that, in particular conditions, a strict link may exist between the endocrine environment and the binding characteristics of mu opioid receptors at hypothalamic level.

## INTRODUCTION

The secretion of the anterior pituitary hormones (e.g. gonadotropins and prolactin) is controlled at hypothalamic level not only by the classical neurotransmitters (e.g. norepinephrine, dopamine, serotonin, etc.), but also by a large series of peptides present in the brain. Among the peptides involved in such a control, a large body of data show that a prevalent role is played by the opioids [1, 2].

In the brain, the naturally occurring opioids (met- and leu-enkephalins, endorphins, dynorphin, etc.) interact with specific binding sites.

*Proceedings of the VIIIth International Congress on Hormonal Steroids*, The Hague, The Netherlands, 16–21 September 1990.

*To whom correspondence should be addressed.

Different classes of opioid receptors (named respectively mu, kappa, delta, etc.) have been described [3]. Among these, the mu receptors appear to be particularly relevant for the control of gonadotropin secretion [4], while the receptors of the mu and kappa families seem to be especially involved in the control of prolactin release [5–6].

Evidence exists suggesting that the endocrine effects of exogenously administered opioids and of their antagonists (e.g. naloxone) may depend on the endocrine status existing at the time of the experiment, and especially on the circulating levels of sex steroids. For example, it has been shown that morphine, a rather specific ligand for the mu receptors, when given into the cerebral ventricles (i.v.t.), increases the release of LH in adult normal male rats, but decreases the secretion of this hormone when given to castrated male rats [7]; moreover, it has also been reported that i.v.t. administered morphine is able to increase serum prolactin levels in adult normal male rats, but is totally ineffective after orchidectomy [8].

The present report will summarize the data obtained in the authors' laboratory on the modifications induced by changing blood levels of sex steroids on the binding characteristics of brain mu opioid receptors in the hypothalamus of the female rat. The emphasis of the present studies was on the opiatergic system of the hypothalamus, because this region of the brain plays a major role in various neuroendocrine processes, and is also particularly rich both in mu opioid receptors and in $\beta$-endorphin, the possible physiological ligand for these receptors [9, 10].

In particular, this paper will describe the changes of the binding characteristics of hypothalamic mu binding sites in experimental models characterized by different patterns of sex steroid levels. The following situations will be considered: (a) the effect of neonatal endocrine manipulations; (b) the effect of estrous cyclicity; (c) the effect of different treatments (performed in ovariectomized female rats) with gonadal steroids able to induce positive or negative feedback effects on gonadotropin secretion; and (d) the effect of pregnancy and of parturition.

In the experiments described, tritiated dihydromorphine (DHM), a typical mu receptor ligand, has been used in a receptor binding assay performed on plasma membrane preparations obtained from hypothalamic tissue, as described in detail elsewhere [11, 12]. The maximal binding capacity ($B_{max}$, expressed as fmol/mg protein) and the dissociation constant ($K_d$) of DHM to mu sites have been evaluated using the program LIGAND developed by Munson and Rodbard [13], adapted for a Macintosh computer, and kindly provided by Dr G. E. Rovati (Milano, Italy).

## EFFECTS OF NEONATAL ENDOCRINE MANIPULATIONS ON THE CONCENTRATION OF HYPOTHALAMIC MU RECEPTORS

The neural mechanisms which control prolactin and gonadotropin secretion are totally different in male and in female mammals [14, 15]; this has also been confirmed by studies in which the opioids and their antagonists have been utilized. For instance, naloxone, a typical opioid antagonist, has been repeatedly found to decrease serum prolactin levels in adult male rats [8], but not in adult females [16, 17]. Moreover, naloxone stimulates LH release in prepubertal female rats, but not in prepubertal male rats [17, 18].

The sex differences in the mechanisms controlling prolactin and gonadotropin secretion have been ascribed to a different pattern of "organization" of the brain in the two sexes.

It is presently recognized that, in the male rat, the neuroendocrine brain develops towards a male pattern of control of prolactin [14] and gonadotropin [15] secretion because it is exposed, during the neonatal period, to the presence of testosterone secreted by the testes. It has also been reported that it is possible to "masculinize" the feminine neuroendocrine brain by administering testosterone to the neonatal female rat, and conversely to "demasculinize" the neuroendocrine male brain by orchidectomizing the male rat immediately after birth [14, 15].

More recently, it has been found that naloxone decreases serum prolactin levels in normal male rats and in neonatally androgenized females, but is ineffective in normal females and in neonatally orchidectomized males [17].

The present experiments have been designed in order to analyze whether, in the rat, changes of the endocrine environment soon after birth could affect the subsequent development of the binding characteristics of hypothalamic mu opioid receptors. It is known that, at least in the rat, the opioid systems are not fully mature at birth and may consequently be manipulated by hormonal treatments (see [19] for references).

The binding characteristics of DHM were evaluated in the hypothalami of: (a) normal male rats; (b) normal female rats; (c) neonatally androgenized (1.25 mg testosterone/rat on day 2 of life) female rats; and (d) neonatally deandrogenized (castration on day 2 of life) male rats. The animals were sacrificed at 16, 26 and 60 days of age.

The results summarized in Table 1 show that, in the rat, a sexual difference exists in the maturation and development of hypothalamic mu receptors. Apparently, at 16 days of age the density of the hypothalamic mu receptors is identical in normal males and females; in females, but not in males, a consistent increase in the concentration of hypothalamic mu receptors occurs between days 16 and 26. Because of this, the concentration of hypothalamic mu receptors at this age is higher in females than in males. This sexual difference is still present at 60 days, when the brain is fully mature. In fact, at 60 days of age, the concentration of mu receptors in normal females remains elevated; moreover, at the age of 60 days, the density of mu sites increases in the hypothalamus of neonatally castrated males to reach the levels found in females. At this time interval, there were no changes in the number of hypothalamic mu receptors in normal males and in androgenized females.

No changes of the affinity of DHM to mu receptors were detected among the different experimental groups.

The present data indicate that the sexual dimorphism in the ontogenesis of hypothalamic mu receptors (as shown by the different pattern of development in normal females vs normal males) may be under the control of the amounts of androgens present at birth. Apparently, the androgens spontaneously present in normal males, or administered to females in the perinatal period, block the development of hypothalamic mu receptors toward a female pattern. The absence of androgens in neonatally castrated male rats permits, on the contrary, the appearance of a female situation, even if at a later stage.

The findings presented here are in agreement with previous studies indicating that endocrine manipulations performed in the neonatal period may modify the development of brain naloxone binding sites in the sexual dimorphic nucleus of the hypothalamic preoptic area [20, 21] as well as the immunoreactivity of met-enkephalin fibers present in the periventricular region of the preoptic area [22].

It remains for further studies to ascertain whether the sexual differences in the concentration of hypothalamic mu receptors found in the present work might explain the differential responses of prolactin and of the gonadotropins to opioid agonists and antagonists previously described to occur in the two sexes [18].

## EFFECT EXERTED BY ESTROUS CYCLICITY ON THE CONCENTRATION OF HYPOTHALAMIC MU OPIOID RECEPTORS

Several data in the literature indicate that, in female animals and in women, the effects of the opioids and of their antagonists on gonadotropin secretion largely depend on the levels of estrogens and progesterone present at time of administration [2, 17, 23].

In a recent experiment performed in the authors' laboratory [24], the modifications of the binding characteristics of mu opioid receptors have been studied in the whole brain of female rats during the different phases of the estrous cycle, which are characterized by different secretory rates of estrogens and progesterone [25, 26]. The results obtained showed that, in the total brain of the female rat, the concentration of mu opioid receptors changes significantly during the different phases of the cycle. In particular, it has been found that the concentrations of mu opioid receptors in the whole brain, which are low during the second day of diestrus, increase during the morning of the day of proestrus reaching their maximum value at 1200 h; after this time there is a

Table 1. Effect of neonatal endocrine environment on the concentration of hypothalamic mu opioid receptors in rats of different ages

| | Days of age | | |
|---|---|---|---|
| Groups | 16 | 26 | 60 |
| Normal males | 103.40 ± 5.95 | 122.46 ± 4.82 | 104.25 ± 12.58 |
| Castrated males | 139.12 ± 18.94 | 122.41 ± 11.89 | 156.46 ± 23.58* |
| Androgenized females | 130.67 ± 14.20 | 107.46 ± 6.91 | 130.14 ± 20.33 |
| Normal females (estrus) | 108.51 ± 7.59 | 168.23 ± 21.53*† | 148.74 ± 12.24*† |

$K_d$ = 0.52 nM. Groups of 6 animals were used. Values of $B_{max}$ (fmol/mg protein) of [$^3$H]DHM are presented as mean ± SE. *Significant ($P < 0.05$) vs normal males of the same age; †significant ($P < 0.05$) vs 16-day old females.

significant and progressive decline of the concentration of mu receptors, which return at 1800 h to the levels found in the early morning of the day of proestrus. During all these studies, the changes in the concentration of mu receptors were not accompanied by any change in the affinity constant.

To complement these data, the present experiments have been performed in order to analyze possible changes of the binding characteristics of opioid receptors of the mu type at the level of the hypothalamus (the region of the brain mainly involved in the control of gonadotropin secretion) during the different phases of the estrous cycle of the female rats. To this purpose different groups of female rats with a regular 4-day estrous cycle were killed by decapitation in different phases of their cycle, i.e. at 1000 h of the first and the second day of diestrus, at 1000, 1200, 1400, 1600, 1800 and 2000 h of the day of proestrus, and at 1000, 1200, 1400, 1600 and 1800 h of the day of estrus. For comparison, female rats ovariectomized 3 weeks before were also used. Serum concentrations of LH, estradiol ($E_2$) and progesterone (P) were measured by RIA to accurately monitor the different phases of the estrous cycle.

It is clear from Fig. 1 (panel A) that the concentration of opioid receptors of the mu type shows significant variations during the different phases of the estrous cycle also in the hypothalamus. The concentration of hypothalamic mu opioid receptors resulted to be of 131.08 fmol/mg protein in the morning of diestrus day 2, showed an increase at 1400 h of the day of proestrus and then started a significant decline with a nadir at 2000 h (68.08 fmol/mg protein; $P < 0.05$) of the day of proestrus. A significant increase of mu sites occurred at 1600 h of the day of estrus. During the other times of observation the values fluctuated around the low levels found at 2000 h of the day of proestrus. The minimum value of the concentration of mu binding sites were recorded in the morning of the first day of diestrus (35.85 fmol/mg protein); the analysis performed by LIGAND detected during diestrus day 1 also a significant increase of the affinity ($K_d$, 0.193 nM) of DHM for the mu hypothalamic receptors when compared to those observed in the second day of diestrus and during the morning of the day of proestrus (ranging from 1.41 to 1.92 nM).

It is interesting to note that ovariectomized female rats show a concentration of hypothalamic mu receptors significantly lower (66.42 fmol/mg protein) than that observed in the second day of diestrus and in the morning of the day of proestrus, but in ovariectomized animals the affinity of DHM is significantly increased (0.342 nM) and similar to that measured in the first day of diestrus.

The present data underline that during the different phases of the estrous cycle the binding characteristics of mu opioid receptors show important changes not only in the whole brain but also in the hypothalamus of the female rat. In fact, the results seem to show a first period of the estrous cycle (second day of diestrus, and morning of proestrus) characterized by a rather high density of hypothalamic mu sites; this is followed by a progressive decline during the afternoon of the day of proestrus and the day of estrus with a minimum value of the concentration of mu receptors recorded in the first day of diestrus. Two small peaks of mu density are observed during the day of estrus.

It is interesting to ask the question whether these estrous cycle-linked modifications of the concentration of brain mu opioid receptors might be considered as primary, or whether they might be induced by the changes of the steroid "milieu" occurring during the different phases of the estrus cycle.

Figure 1 (panel B and C) shows the plasma levels, measured by RIA, of LH, $E_2$ and P detected during the experiment. It is evident that the decline of mu opioid receptors in the hypothalamus during the afternoon of the day of proestrus seems to be coincident with the LH surge. It is also apparent from Fig. 1 that the higher concentrations of hypothalamic mu receptors are present during the period of the estrous cycle characterized by high serum levels of $E_2$, and that the decrease of the density of the mu sites starts concomitantly with the increase of P secretion. Moreover, in the hypothalamus of ovariectomized animals, there is a low concentration of mu binding sites accompanied by an increase of the affinity similar to that observed during the first day of diestrus, when both $E_2$ and P are at low levels. The data would then imply that the changes of the concentration of the hypothalamic mu receptors are linked to, if not induced by, the modifications of serum levels of both $E_2$ and P. However, the fluctuation of opioid receptors occurring during the day of estrus appears difficult to explain on the basis of changes of sex steroid secretion.

The results reported here are in agreement with the studies which have shown that, during the preovulatory surge of LH, the LH-releasing response of rats to naloxone is reduced or abolished [17, 27] suggesting a decrease of the opiatergic inhibitory tone on the secretion of LH [2]. The results are in contrast with those of Weiland and Wise [28] who, using an autoradiographic procedure, have reported no modifications of naloxone binding sites in discrete regions of the hypothalamus of female rats during the day of proestrus. Moreover, in contrast with the present data these authors [28] have found a significant increase of hypothalamic naloxone binding in 7-day ovariectomized rats vs the values observed at proestrus in intact animals. This discrepancy may be due to the different interval after castration (3 weeks in the present study vs 7 days in the study of Weiland and Wise [28]).

In conclusion, the present data indicate that in the female rat, in a physiological situation, a link seems to exist between serum levels of sex steroids and the density of hypothalamic mu opioid receptors.

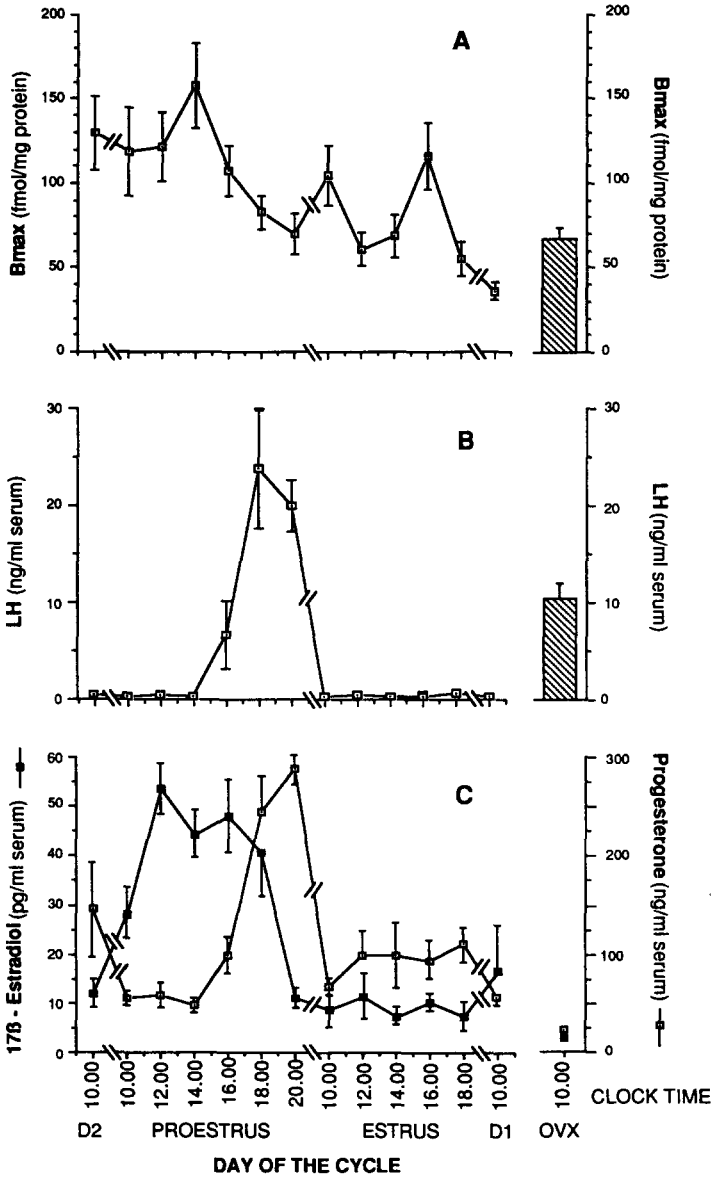


Fig. 1. Concentration of hypothalamic mu opioid receptors (panel A) and serum LH (panel B), 17$\beta$-estradiol and progesterone (panel C) during the various phases of the estrous cycle of female rats. (Groups of 10 animals were used; values represent mean ± SE; OVX: 3-week ovariectomized animals.)

## EFFECTS OF SEX STEROID TREATMENT ON THE CONCENTRATION OF HYPOTHALAMIC MU RECEPTORS

This series of experiments were designed in order to evaluate whether steroid regimes able to elicit negative or positive feedback effects on LH secretion might modify the concentration of hypothalamic mu opioid receptors in adult castrated female rats.

Adult female rats, ovariectomized 3 weeks before, have been treated according to the following schedules: (a) short-term treatment—ovariectomized animals were injected s.c. with two doses (7.5 $\mu$g/rat) of estradiol benzoate (EB) in the morning of experimental days 1 and 2 and sacrificed 6 h after the second injection of EB; (b) long-term treatment—Silastic capsules filled with crystals of 17$\beta$-estradiol free base ($E_2$) were implanted s.c. in ovariectomized animals; these were sacrificed two weeks after $E_2$ implantation; these are standard procedures known to induce a negative feedback effect on gonadotropin release [26]; (c) finally, ovariectomized female rats were treated s.c. with 7.5 $\mu$g/rat of estradiol benzoate in the morning of experimental day 1 and with 5 mg/rat of progesterone on the morning of experimental day 3. The animals were sacrificed 6 h after the administration of progesterone. This type of treatment is known to induce a positive feedback effect on LH secretion [26]. At the end of each experiment, serum levels of LH were measured by radioimmunoassay concomitantly with the evaluation of the concentration of hypothalamic mu binding sites.

As expected, both short-term and long-term estrogen treatment induced a significant decrease of serum LH levels, while the combined treatment with estrogen plus progesterone induced a significant increase in serum LH levels (Table 2).

Table 2. Effect of gonadal steroid treatment on the concentration of hypothalamic mu opioid receptors in 3-week ovariectomized (OVX) female rats

| Treatment | LH (ng/ml) | Hypothalamic mu sites ($B_{max}$; fmol/mg protein) |
|---|---|---|
| OVX | 10.66 ± 2.02 | 117.22 ± 15.24 |
| EB | 4.19 ± 0.35* | 108.97 ± 12.97 |
| OVX | 13.02 ± 1.67 | 118.12 ± 4.93 |
| $E_2$ | 3.11 ± 0.37* | 112.76 ± 6.35 |
| OVX | 16.74 ± 0.78 | 106.20 ± 5.65 |
| EB-P | 39.43 ± 6.22* | 90.14 ± 3.33* |

$K_d$ = 1.12 nM. Groups of 10 animals were used. Values represent mean ± SE. *Significant ($P < 0.05$) vs vehicle-treated OVX of the same group; EB = estradiol benzoate; $E_2$ = 17$\beta$-estradiol; P = progesterone.

Table 2 also shows that in the female rats treated only with estrogens, both in the short-term or in the long-term experiment, there are no apparent changes in the concentrations of the hypothalamic mu opioid receptors, vs the ovariectomized vehicle-treated control animals. In no instance were there changes of the dissociation constant ($K_d$) of the DHM to mu sites. These results suggest that the negative feedback effect of estrogens on LH release probably does not involve alterations of the hypothalamic mu receptors.

Moreover, it is evident from Table 2 that the treatment with estrogen plus progesterone brings about a significant decrease of the concentration of dihydromorphine binding sites in the hypothalamus vs that found in control animals. Also in this experiment, there were no changes of the affinity. These data are compatible with the hypothesis that the positive feedback effect of estrogen plus progesterone on LH secretion might be due to changes in the concentration of hypothalamic mu binding sites.

Several investigators have analyzed the effects of treatment with estrogens on opioid receptor binding. In agreement with the findings here reported, Jacobson and Kalra [29] reported that the acute treatment of 3 week-ovariectomized female rats with EB does not modify the binding of tritiated naloxone, an opiate antagonist, in the mediobasal hypothalamus and in the preoptic area. On the contrary, some authors have demonstrated a significant increase in naloxone binding in the anterior hypothalamus of ovariectomized female rats following more prolonged (12 weeks, [30]; 2 weeks, [31]) estrogen treatment through the implantation of Silastic capsules filled with estrogens. The present data are in contrast with those of Weiland and Wise [28] who have recently reported that the implantation of Silastic capsules filled with $E_2$ for 2 days induces a decrease of naloxone binding in discrete regions of the hypothalamus of 7-day ovariectomized female rats.

In the authors' opinion, all these data cannot be directly compared with those reported in this paper because of the differences in the experimental models used (e.g. different type and duration of treatments, time of recovery after castration, autoradiography vs biochemical assay, etc.); moreover, all these authors used naloxone a ligand, which also binds to other kinds of opioid receptors [9]; in the present experiments, on the contrary, dihydro-

morphine was used, which is a rather specific ligand for opioid mu receptors.

It must be pointed out, however, that a general agreement seems to exist on the fact that the treatment with estrogens plus progesterone, able to produce a positive feedback effect on LH secretion, induces a significant decrease of the opioid receptors in different regions of the hypothalamus in ovariectomized rats [28, 29].

All these data are compatible with the hypothesis proposed here that the positive feedback effect of estrogen plus progesterone on LH secretion might be due to a decrease of the concentration of hypothalamic mu binding sites and consequently to a decrease of the opiatergic tone bringing to a gradual decrease of the inhibitory influences on LH secretion; this may obviously facilitate the occurrence of the LH surge [2].

## EFFECTS EXERTED BY PREGNANCY AND PARTURITION ON HYPOTHALAMIC MU OPIOID RECEPTORS

Serum levels of pituitary and gonadal hormones show marked changes during pregnancy and at parturition [14, 32–34]. The concentrations of $\beta$-endorphin in the brain have also been shown to change during pregnancy and lactation [35]. Because of this, it was found of interest to analyze the binding characteristics of the mu subpopulation of opioid receptors in the hypothalamus of female rats at different stages of pregnancy, on the day of parturition and, during the postpartum period, both in lactating and in non-lactating animals. In particular, the mu receptors have been measured in the hypothalamus of female rats killed on day 7 or on day 15 of pregnancy, on the day of parturition (day 22), as well as 12–18 h and 6–8 days after delivery. At the last time interval, the data were obtained from females that had been suckled by their pups, and from those that had been separated from their offspring immediately after parturition. Cycling female rats killed in the day of estrous served as controls. The results obtained (Table 3) show that the hypothalamic concentration of mu opioid receptors was significantly higher than in controls on day 15 of pregnancy and on the day of parturition; after delivery the concentration of mu receptors returned to control values, irrespective of whether the measurement was performed in lactating or in non-lactating animals. In no instances there were variations of the $K_d$ of DHM to mu receptors.

Table 3. Hypothalamic concentration of mu opioid receptors during pregnancy and after parturition in female rats

| | |
|---|---|
| Controls (estrus) | 180.32 ± 15.17 |
| 7 Days | 225.63 ± 23.70 |
| 15 Days | 272.66 ± 26.95* |
| 22 Days | 273.53 ± 18.92* |
| 12–18 h Postpartum | 189.75 ± 24.14 |
| 6–8 days Postpartum (non-lactating) | 226.61 ± 18.34 |
| 6–8 days Postpartum (lactating) | 261.01 ± 20.30 |

$K_d = 0.8$ nM. Groups of 6 animals were used. Values of $B_{max}$ (fmol/mg protein) are presented as mean ± SE. *Significant ($P < 0.05$) vs controls.

The data presented here show that the hypothalamic concentration of mu opioid receptors significantly increases throughout pregnancy and returns towards control levels during the postpartum period. The increase in the density of opioid receptors reported here to occur in the hypothalamus of pregnant rats seems to indicate that the hypothalamic opiatergic tone markedly increases during pregnancy. This enhanced opiatergic activity may be either primary, or intervene as the consequence of several factors. These may include: (a) the changes of the gonadal steroid environment which characterize this particular physiological condition [34, 36–40]; and (b) the preparation for the stress of parturition.

In order to ascertain whether the modifications of the density of mu receptors observed during pregnancy may be due to the changing levels of sex steroids, additional experiments were performed. Adult 7-day ovariectomized female rats were implanted subcutaneously with Silastic capsules filled with $E_2$ and capsules filled with P; this treatment was able to produce serum levels of these steroids comparable to those found during the first half of pregnancy ($E_2$, 30 pg/ml; P, 220 ng/ml). A second group of animals received only capsules filled with estradiol. Animals were killed on day 14 of treatment. Control groups included ovariectomized rats implanted with empty Silastic capsules and normal cycling female rats killed on the day of estrus.

As illustrated in Table 4, treatment with $E_2$ alone induced a decrease of the secretion of LH. In contrast with the positive feedback effect on LH secretion exerted by the acute EB + P treatment (see Table 2), the long-term treatment with $E_2$ + P performed in this experiment produced a significant decrease of plasma LH levels. This result is in line with the finding of a decrease of LH levels during middle pregnancy (data not shown).

Table 4 clearly shows that the treatments with $E_2$ and $E_2$ + P were unable to induce any

Table 4. Effect of gonadal steroid treatment on the concentration of hypothalamic mu opioid receptors in 7-day ovariectomized (OVX) female rats

| Treatment | LH (ng/ml) | Hypothalamic mu sites ($B_{max}$; fmol/mg protein) |
|---|---|---|
| OVX | 8.145 ± 1.24 | 152.36 ± 35.04 |
| $E_2$ | 0.918 ± 0.29* | 171.80 ± 17.20 |
| $E_2$ + P | 0.102 ± 0.02* | 149.30 ± 16.42 |

$K_d$ = 1.32 nM. Groups of 10 animals were used. Values represent mean ± SE. *Significant ($P < 0.05$) vs vehicle-treated OVX animals. $E_2$ = 17β-estradiol; P = progesterone.

modification of the concentration of hypothalamic mu opioid receptors or to modify the affinity of DHM to the mu sites (both when the data were analyzed vs those in ovariectomized rats or in females killed on the day of estrus). There are no previous data which may be directly compared with those of the two studies here reported. However, in partial agreement with the present observations, Hammer and Bridges [31] have reported that the concentration of [$^3$H]naloxone binding sites is particularly high in the medial preoptic area of the hypothalamus (MPOA) of pregnant rats (day 12 of gestation), and shows a significant decline in lactating animals 12 days after delivery.

These authors [31] also reported a greater binding of naloxone in the MPOA of $E_2$ + P-treated rats than in ovariectomized animals. Methodological differences (binding assay, type of ligand used, brain region studied, etc.) must be considered to explain these discrepancies.

On the basis of the present results the authors would then be inclined to suggest that, during pregnancy, the concentration of hypothalamic mu opioid receptors is not linked to endocrine phenomena, but might be related to the stressful conditions represented by pregnancy itself and by the oncoming delivery. Brain opioid peptides have been actually demonstrated to be significantly affected by stressful stimuli of various nature [40–43].

One might also postulate that the hypothalamic system of opiate receptors may play a major physiological role during pregnancy and parturition by regulating maternal pain perception. It has been clearly shown that the pain threshold is significantly increased in pregnant rats, and that this effect can be abolished by treatment with the opiate antagonist naloxone [44].

Moreover, it has been shown that maternal behavior, which develops during lactation, is related not only to gonadal steroid levels [38, 45] but also to opiatergic inputs [46, 47]. Thus, the decreased opiatergic activity observed in the hypothalamus of lactating rats might be necessary for the onset of maternal behavior. Endogenous opioids are also known to inhibit oxytocin release [48]; for instance, the administration of morphine to lactating mice and rats has been shown to decrease the weight gain of the pups during suckling [49, 50]. Therefore, it is possible that the decrease in hypothalamic opioid activity during lactation might be directed to facilitating oxytocin release and therefore to promoting lactation.

*Acknowledgements*—The experiments performed in the authors' laboratory and here reported were supported by grants of the Consiglio Nazionale delle Ricerche (through the Special Projects "FATMA" No. 91.00207 PF41, "AGING" No. 91.00419 PF40, "Biotechnology and Bioinstrumentation" No. 91.0192 PF20 and Bilateral Project No. 91.00303 CT04) and by funds of the MURST. Materials for LH RIA have been kindly provided by the Rat Pituitary Hormone Distribution Program of the NIADDK (Bethesda, Md). Thanks are also due to Mrs Paola Assi for her skilful technical assistance.

## REFERENCES

1. Meites J., Bruni J. F., Van Vugt D. A. and Smith A. F.: Relation of endogenous opioid peptides and morphine to neuroendocrine functions. *Life Sci.* **24** (1979) 1325–1336.
2. Kalra S. P.: Neural circuitry involved in the control of LHRH secretion: a model for preovulatory LH release. In *Frontiers in Neuroendocrinology* (Edited by W. F. Ganong and L. Martini). Raven Press, New York (1986) pp. 31–75.
3. Paterson S. J., Robson L. E. and Kosterlitz H. W.: Classification of opioid receptors. *Br. Med. Bull.* **39** (1983) 31–36.
4. Pfeiffer D. G., Pfeiffer A., Shimohigashi Y., Merriam G. R. and Loriaux D. L.: Predominant involvement of mu- rather than delta- or kappa-opiate receptors in LH secretion. *Peptides* **4** (1983) 647–649.
5. Spiegel K., Kourides I. A. and Pasternak G. W.: Different receptors mediate morphine-induced prolactin and growth hormone release. *Life Sci.* **31** (1982) 2177–2180.
6. Krulich L., Koenig J. I., Conway S., McCann S. M. and Mayfield M. A.: Opioid kappa receptors and the secretion of prolactin (Prl) and growth hormone (GH) in the rat. *Neuroendocrinology* **42** (1986) 75–87.
7. Piva F., Limonta P., Maggi R. and Martini L.: Stimulatory and inhibitory effects of the opioids on gonadotropin secretion. *Neuroendocrinology* **42** (1986) 504–512.
8. Limonta P., Piva F., Maggi R., Dondi D., Motta M. and Martini L.: Morphine stimulates prolactin release in normal but not in castrated male rats. *J. Reprod. Fert.* **76** (1986) 745–750.
9. Chang K. J., Cooper B. R., Hazum E. and Cuatrecasas P.: Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. *Molec. Pharmac.* **16** (1979) 91–104.
10. Cuello A. C.: Central distribution of opioid peptides. *Br. Med. Bull.* **39** (1983) 11–16.
11. Piva F., Maggi R., Limonta P., Dondi D. and Martini L.: Decrease of mu opioid receptors in the brain and in the hypothalamus of the aged male rat. *Life Sci.* **40** (1987) 391–398.
12. Maggi R., Limonta P., Dondi D., Martini L. and Piva F.: Distribution of kappa opioid receptors in the

brain of young and old male rats. *Life Sci.* **45** (1989) 2085–2092.
13. Munson P. J. and Rodbard D.: LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Analyt. Biochem.* **107** (1980) 220–239.
14. Neill J. D. and Smith M. S.: Pituitary-ovarian interrelationships in the rat. In *Current Topics in Experimental Endocrinology* (Edited by V. H. T. James and L. Martini). Academic Press, New York, Vol. II (47) (1974) pp. 73–106.
15. Gorski R. A.: Sexual differentiation of the brain: possible mechanisms and implications. *Can. J. Physiol. Pharmac.* **63** (1985) 577–594.
16. Piva F., Maggi R., Limonta P., Motta M. and Martini L.: Effect of naloxone on luteinizing hormone, follicle-stimulating hormone and prolactin secretion in the different phases of the estrous cycle. *Endocrinology* **117** (1985) 766–772.
17. Limonta P., Dondi D., Maggi R., Martini L. and Piva F.: Neonatal organization of the brain opioid system controlling prolactin and luteinizing hormone secretion. *Endocrinology* **124** (1989) 681–686.
18. Blank M. S., Panerai A. E. and Friesen H. G.: Opioid peptides modulate luteinizing hormone secretion during sexual maturation. *Science* **203** (1979) 1129–1131.
19. Kornblum H. I., Hurblut D. E. and Leslie F. M.: Postnatal development of multiple opioid receptors in rat brain. *Dev. Brain Res.* **37** (1987) 21–41.
20. Hammer R. P. Jr.: The sexually dimorphic region of the preoptic area in rats contain denser opiate receptors binding sites in females. *Brain Res.* **308** (1984) 172–176.
21. Hammer R. P. Jr.: The sex hormone-dependent development of opiate receptors in the rat medial preoptic area. *Brain Res.* **360** (1985) 65–74.
22. Watson R. E. Jr., Hoffman G. E. and Wiegand S. J.: Sexually dimorphic opioid distribution in the preoptic area: manipulation by gonadal steroids. *Brain Res.* **398** (1986) 157–163.
23. Quigley M. E. and Yen S. S. C.: The role of endogenous opiates on LH secretion during the menstrual cycle. *J. Clin. Endocr. Metab.* **51** (1980) 179–181.
24. Casulari L. A., Maggi R., Dondi D., Limonta P., Piva F., Motta M. and Martini L.: Effect of oestrus cyclicity on the number of brain opioid mu receptors in the rat. *Horm. Metab. Res.* **19** (1987) 549–554.
25. Barraclough C. A., Collu R., Massa R. and Martini L.: Temporal interrelationships between plasma LH, ovarian secretion rates and peripheral plasma progestin concentrations in the rat: effects of nembutal and exogenous gonadotropins. *Endocrinology* **118** (19) 1437–1447.
26. Kalra S. P. and Kalra P. S.: Neural regulation of luteinizing hormone secretion in the rat. *Endocrine Rev.* **4** (1983) 311–351.
27. Gabriel S. M., Simpkins J. W. and Kalra S. P.: Modulation of endogenous opioid influence on luteinizing hormone secretion by progesterone and estrogen. *Endocrinology* **113** (1983) 1806–1811.
28. Weiland N. G. and Wise P. M.: Estrogen and progesterone regulate opiate receptor densities in multiple brain regions. *Endocrinology* **126** (1990) 804–808.
29. Jacobson W. and Kalra S. P.: Decrease in mediobasal hypothalamic and preoptic area opioid ([$^3$H]Naloxone) binding are associated with the progesterone-induced luteinizing hormone surge. *Endocrinology* **124** (1989) 199–206.
30. Wilkinson M., Brawer J. R. and Wilkinson D. A.: Gonadal steroid-induced modification of opiate binding sites in anterior hypothalamus of female rats. *Biol. Reprod.* **32** (1985) 501–506.
31. Hammer R. P. Jr. and Bridges R. S.: Preoptic area opioids and opiate receptors increase during pregnancy and decrease during lactation. *Brain Res.* **420** (1987) 48–56.
32. Amenomori Y., Chen C. L. and Meites J.: Serum prolactin levels in rats during different reproductive states. *Endocrinology* **86** (1970) 506–510.
33. Linkie D. M. and Niswender G. D.: Serum levels of prolactin, luteinizing hormone, and follicle stimulating hormone during pregnancy in the rat. *Endocrinology* **90** (1972) 632–637.
34. Bridges R. S.: A quantitative analysis of the roles of dosage, sequence and duration of estradiol and progesterone exposure in the regulation of maternal behavior in the rat. *Endocrinology* **114** (1984) 930–940.
35. Wardlaw S. L., Thoron L. and Frantz A. G.: Effects of sex steroids on brain $\beta$-endorphin. *Brain Res.* **245** (1982) 327–331.
36. Sanyal M. K.: Secretion of progesterone during gestation in the rat. *J. Endocr.* **79** (1978) 179–180.
37. Taya K. and Greenwald G. S.: *In vivo* and *in vitro* ovarian steroidogenesis in the pregnant rat. *Biol. Reprod.* **114** (1981) 930–940.
38. Gallo R. V., Devorshak-Harvey E. and Bona-Gallo A.: Pulsatile luteinizing hormone release during pregnancy in the rat. *Endocrinology* **116** (1985) 2637–2642.
39. Devorshak-Harvey E., Bona-Gallo A. and Gallo R. V.: The relationship between declining plasma progesterone levels and increasing luteinizing hormone pulse frequency in late gestation in the rat. *Endocrinology* **120** (1987) 1597–1601.
40. Millan M. J., Przewlocki R., Jerlicz M., Gramsch C. H., Hollt V. and Herz A.: Stress-induced release of brain and pituitary $\beta$-endorphin: major role of endorphins in generation of hyperthermia, not analgesia. *Brain Res.* **208** (1981) 325–338.
41. Maggi R., Dondi D., Panerai A. E. and Limonta P.: Hypothalamic $\beta$-endorphin and brain mu opiate receptor concentrations in male rat after restraint stress. *Neuroendocr. Lett.* **10** (1988) 289–295.
42. Lim A. T. W. and Funder J. W.: Stress-induced changes in plasma, pituitary and hypothalamic immunoreactive $\beta$-endorphin: effects of diurnal variation, adrenalectomy, corticosteroids and opiate agonists and antagonists. *Neuroendocrinology* **36** (1983) 225–234.
43. Kant G. J., Mougey E. H. and Meyerhoff J. L.: Diurnal variation in neuroendocrine response to stress in rats: plasma ACTH, $\beta$-endorphin, $\beta$-LPH, corticosterone, prolactin and pituitary cyclic AMP responses. *Neuroendocrinology* **43** (1986) 383–390.
44. Gintzler A. R.: Endorphin-mediated increases in pain threshold during pregnancy. *Science* **210** (1980) 193–195.
45. Rosenblatt J. S., Mayer A. D. and Giordano A. L.: Hormonal basis during pregnancy for the onset of maternal behavior in the rat. *Psychoneuroendocrinology* **13** (1988) 29–46.
46. Grimm C. T. and Bridges R. S.: Opiate regulation of maternal behavior in the rat. *Biochem. Behav. Pharmac.* **19** (1983) 609–616.
47. Numan M.: Neural basis of maternal behavior in the rat. *Psychoneuroendocrinology* **13** (1988) 47–62.
48. Leng G., Mansfield S., Bicknell R. J., Dean A. D. P., Ingram C. D., Marsh M. I. C., Yates J. O. and Dyer R. G.: Central opioids: a possible role in parturition? *J. Endocr.* **106** (1985) 219–224.
49. Haldar J. and Sawyer W. H.: Inhibition of oxytocin release by morphine and its analogs. *Proc. Soc. Exp. Biol. Med.* **157** (1978) 476–480.
50. Russell J. A. and Spears N.: Morphine inhibits suckling induced oxytocin secretion in conscious lactating rats but also disrupts maternal behavior. *J. Physiol.* **346** (1984) 133P.