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Effects of Steroids on the Brain Opioid System

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The experiments reported here add further evidence in support of the view that sex steroids may influence the binding characteristics of brain opioid receptors. In particular, it has been shown that: (a) the number of μ-opioid receptors varies in the hypothalamus of regularly cycling female rats according to the different phases of the estrous cycle, which are characterized by fluctuations of circulating levels of sex steroids; (b) the number of μ-opioid receptors decreases in the hypothalamus and in the corpus striatum when ovariectomized rats are submitted to treatments with estradiol and progesterone able to induce a "positive" feedback effect on LH release. A treatment with estrogen alone able to induce a "negative" feedback effect on LH release brings about an increase of the number of μ-opioid receptors in the thalamus and in the hippocampus; (c) in addition to the μ-receptors, receptors of the delta type may also be involved in the control of gonadotropin secretion; recent results here presented indicate that a line of immortalized hypothalamic cells (GT1 cells), which synthesize and secrete LHRH, present δ opioid receptors on their membranes; these are apparently involved in the control of LHRH release from these cells.

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INTRODUCTION

The regulation of the secretion of pituitary gonadotropins is the result of a complex interplay between the feedback effects of gonadal steroids and the influence exerted by brain neurotransmitters on the hypothalamic–pituitary complex [1, 2]. A central role in these phenomena is played by brain opioids, which mainly exert an inhibitory influence on gonadotropin secretion [2].

It is known that the different classes of brain opioids (β-endorphin, enkephalins, dynorphin, etc.) exert their actions through binding to specific membrane receptors named respectively μ, δ, and κ [3]. The receptors of the μ type have been believed until recently to be those playing the major role in the control of gonadotropin secretion [4]. Some new data reported below will indicate that opioid receptors of the delta type are also of importance in gonadotropin regulation.

Many reports suggest that the effects of exogenously administered opioids, of their agonists and of their antagonists on gonadotropin secretion depend upon the endocrine status existing at the time of the experiment, and more particularly on the circulating levels of sex steroids (see [2] and [5] for references). The possibility exists that such variations in the responses of gonadotropins to opioid agonists and antagonists are due to alterations of the binding characteristics of opioid receptors induced by the changes in the endocrine "milieu".

The present report summarizes the data collected in this laboratory concerning the modifications induced by changing blood levels of sex steroids on the binding characteristics of brain μ-opioid receptors in the female rat. The following issues will be discussed: (a) effect of estrous cyclicity; (b) effect of different treatments with gonadal steroids able to induce positive or negative feedback effects on gonadotropin secretion; and (c) in addition, data suggesting a direct opioid control on LHRH secreting neurons via receptors of the delta type will be presented.

EFFECT OF ESTROUS CYCLICITY

It has been observed that, in the whole brain of the female rat, the number of the opioid receptors of the μ type fluctuates during the different phases of the estrous cycle [6], a phenomenon which may explain the different magnitude of the effects exerted on

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gonadotropin release by opioid agonists and antagonists in the different phases of the ovulatory cycle (see [2] and [5] for references). A study has been devoted to analyze: (1) the concentrations of μ-opioid receptors in the hypothalamus of female rats in the different phases of the estrous cycle using a specific μ-receptor ligand; and (2) to correlate the possible changes of hypothalamic μ-opioid receptors to serum profiles of ovarian steroids and pituitary gonadotropins. To this purpose, different groups of adult female rats with a regular 4-day estrous cycle have been killed in the morning of diestrus days 1 and 2 and, at 2 h intervals, during the days of proestrus and estrus. The hypothalami have been collected, and the binding characteristics (B_{max} and K_d) of dihydromorphine (DHM), a specific μ ligand, on μ-receptors have been evaluated in plasma membrane preparations. Serum levels of LH, estradiol and progesterone have been measured throughout the experiment.

The data obtained in this laboratory [7] show that, as expected from previous findings from this laboratory, the LH surge took place between 16.00 and 20.00 h of the day of proestrus with a peak value at 18.00 h. Estradiol started to rise at 10.00 h of proestrus, reached the maximal concentration at 12.00 h, and then presented a plateau lasting up to 18.00 h. By 20.00 h estradiol was again at basal levels. Progesterone began to rise at 16.00 h of the day of proestrus, peaked at 20.00 h and returned to basal levels by 10.00 h of the day of estrus (Fig. 1).

In the hypothalamus of female rats with a regular 4-day estrous cycle the binding characteristics of DHM for μ-opioid receptors show important variations during the different phases of the estrous cycle (Fig. 2). In general, the data have indicated that the number of μ-opioid receptors is elevated during the days of diestrus day 2 and of proestrus. On proestrus the number of μ-receptors is maximal at 14.00 h, significantly declines at 18.00–20.00 h of the same day, and is low at 12.00, 14.00 and 18.00 h of the day of estrus; the lowest levels have been found in the morning of diestrus day 1. The variations observed during proestrus took place without any significant modifications of the affinity of the ligand for μ-receptors.

It is interesting to underline that the results here reported indicate a progressive decline of the number of hypothalamic μ-receptors in the afternoon of proestrus which coincides with the initiation of the LH surge. It is possible that the decline of the number of hypothalamic μ-receptors at this time might be indicative of a diminished opiatergic tone, and that this might be influential in facilitating the release of LHRH. It is known that opioid agonists usually inhibit LHRH and LH release [2]. The authors do not have a clear-cut explanation for the decline of the number of hypothalamic μ-receptors during this phase of the estrous cycle. This might be primitive, or might be secondary to the increase in the serum levels of estradiol and progesterone occurring at this time interval. The latter interpretation might find support in recent observations indicating that the number of hypothalamic μ-opioid receptors decreases in ovariectomized estrogen-primed rats treated with progesterone according to protocols able to induce an LH surge (see below; see also [2] and [8] for references). The pattern reported here on the density of μ-receptors occurring in the

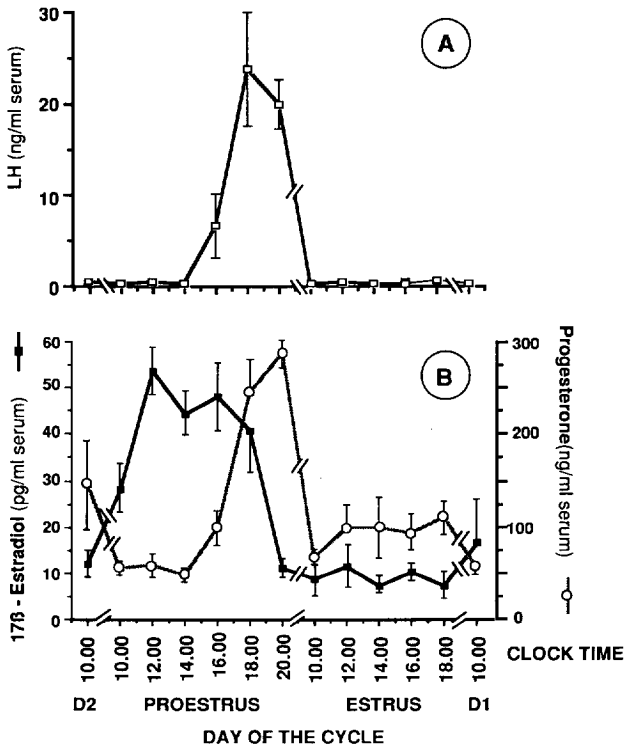

Fig. 1. Serum levels of LH (A) and of estradiol and progesterone (B) of adult regularly cycling female rats killed in different phases of the estrous cycle.

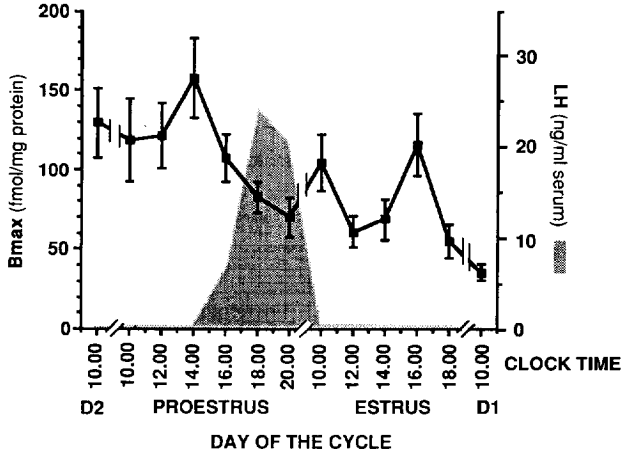

Fig. 2. Variations of the concentration of μ-opioid receptors in the whole hypothalamus of adult regularly cycling female rats killed in different phases of the estrous cycle. For reference, serum levels of LH detected at the same time intervals are reported (shadowed area). D2 = second day of diestrus; D1 = first day of diestrus.

afternoon of proestrus may also explain the decrease, or the absence, of the effects of opioid agonists and antagonists during spontaneous or steroid-induced gonadotropin surges, as reported by several groups (see [2] and [5] for references). However, the fluctuations of the number of μ-receptors during the day of estrus seem difficult to be linked to circulating levels of sex steroids, since these variations were observed in the presence of uniformly low serum levels of estrogen and progesterone.

To further clarify this issue a second set of experiments has been performed in order to clarify whether sex steroids indeed affect the binding characteristics of μ-receptors present in the hypothalamus and in extrahypothalamic structures not previously considered.

EFFECT OF DIFFERENT TREATMENTS WITH GONADAL STEROIDS ABLE TO INDUCE POSITIVE OR NEGATIVE FEEDBACK EFFECTS ON GONADOTROPIN SECRETION

The experiments to be reported here have been planned in order to analyze whether, in castrated female rats, opioid binding sites of the μ-type are present not only in the hypothalamus but also in the extrahypothalamic encephalon, and whether their density can be affected by experimental changes of circulating levels of sex steroids. The protocol adopted has been that of evaluating the binding characteristics of μ-binding sites in circumscribed brain areas of adult ovariectomized rats subjected to treatments with estrogens and progesterone. Two paradigms have been employed: (1) a treatment with estrogens plus progesterone able to induce an increase of gonadotropin secretion (positive feedback effect); and (2) a treatment with estrogens only able to induce a decrease of gonadotropin secretion (negative feedback effect). The areas selected have been: frontal poles, anterior cerebral cortex, posterior cerebral cortex, corpus striatum, amygdala, hypothalamus, mesencephalon, hippocampus, thalamus.

The data obtained in this laboratory [8] first describe the distribution of the μ-opioid binding sites in different areas of the brain of adult castrated female rats. It is apparent from Figs 3 and 4 that the number of μ-receptors is consistently high in the frontal poles, in the anterior cerebral cortex and in the corpus striatum, tends to decrease in the posterior cerebral cortex, in the thalamus, in the amygdala and in the hypothalamus, and presents the lowest values in the hippocampus and in the mesencephalon. The affinity of the ligand for the receptors was similar in all the areas considered. The present data also show that the treatment of female rats ovariectomized for 3 weeks with estradiol benzoate (EB) plus progesterone (P), which induces a positive feedback effect on LH release, significantly decreases the number of μ-binding sites

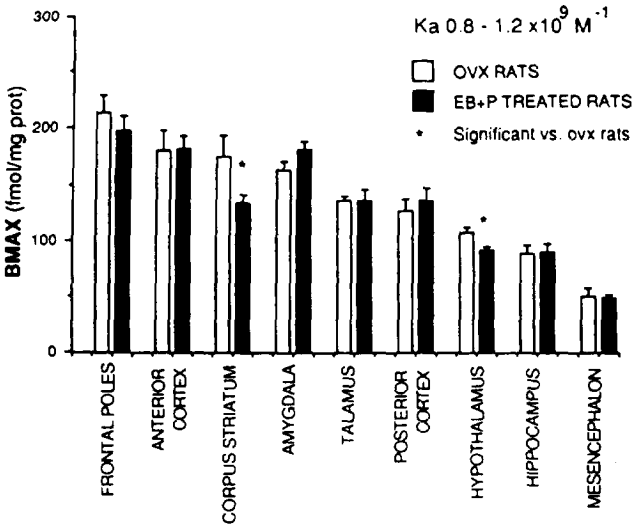

Fig. 3. Effect of the treatment with estradiol benzoate (EB) + progesterone (P) on the binding characteristics of μ opioid receptors in different brain areas of adult ovariectomized (OVX) rats.

in the hypothalamus and in the corpus striatum (CS) (Fig. 3). The effect observed at the level of the hypothalamus is in good agreement with recent results obtained in other laboratories using different experimental protocols and assay methods [9, 10]. If one assumes that a decrease in the number of opioid binding sites at the level of a brain structure is an index of a decrease of the opiatergic tone impinging on that structure, the findings presented here [8], as well as those from other laboratories [9, 10], might suggest that the positive feedback effect exerted by the estradiol–progesterone treatment on gonadotropin

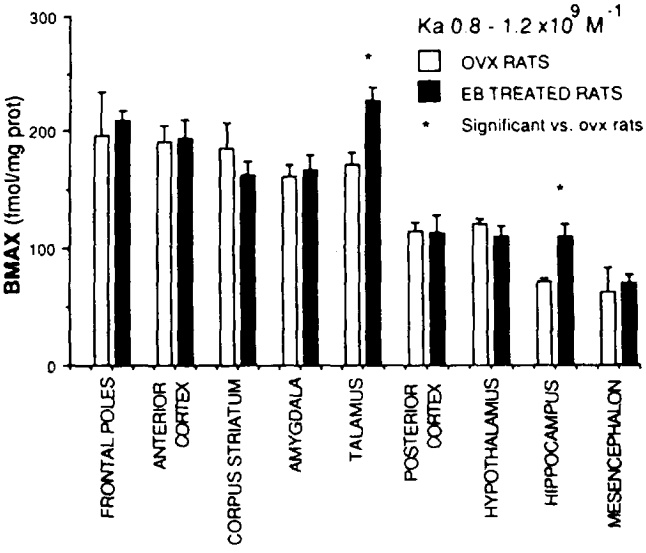

Fig. 4. Effect of the treatment with estradiol benzoate (EB) on the binding characteristics of μ-opioid receptors in different areas of adult ovariectomized (OVX) rats.

release is linked to a decrease of the inhibitory opiatergic tone which usually keeps gonadotropin secretion in check.

The finding that the EB-P treatment adopted in the present experiments induces a decrease in the number of μ-binding sites at the level of the CS was not completely unexpected. Several data show in fact that many extrahypothalamic structures, including the CS, may be the target for gonadal hormones (see [11] for a review). On the other hand, it may be recalled that a report published some years ago by Carrillo [12] suggests that the CS may exert a stimulatory role in the regulation of gonadotropin secretion: according to this author large lesions of the caudate-putamen complex bring about, in female rats, a significant decrease of the proestrus LH surge and of the number of ova shed.

In the present study it has also been shown that a treatment with EB alone increased the number of DHM binding sites in the hippocampus and thalamus (Fig. 4), without modifying the receptorial status of other brain regions. The observed increase in the number of μ-binding sites in the hippocampus and thalamus might indicate that an increase of the opiatergic inhibitory tone occurs at the level of these areas under the influence of estrogens. With regard to the hippocampus, it is known that this structure is a target for estrogens [13], and that it may exert an inhibitory influence on gonadotropin release (see [14] for a review). Consequently, the decrease of serum levels of LH brought about by the EB treatment might be due, at least in part, to the activation of opioid systems in this structure. No data are available on a possible interplay between the thalamus and the nervous circuitries controlling gonadotropin secretion. However, it might be of interest to recall that also thalamic opioid systems are sensitive to circulating levels of gonadal steroids. Wardlaw *et al.* [15] have reported that castrated females exposed for 3 weeks to estrogens exhibit a decrease of thalamic endorphin content. The interaction of steroids and opioid systems in the thalamus might be relevant for some somatosensory function not related to endocrine phenomena.

The lack of effect of estradiol on hypothalamic μ-opioid receptors seems to suggest that a variation in the density of hypothalamic μ-binding sites is not crucial for the display of the negative feedback effect induced by estrogen alone; the data suggest, however, that the opiatergic systems in the hippocampus may play some role in such a phenomenon.

POSSIBLE DIRECT OPIOID CONTROL ON LHRH SECRETING NEURONS VIA RECEPTORS OF THE DELTA TYPE

As mentioned in the Introduction, several studies have shown that the neurons which synthesize LHRH are innervated by multiple neuronal pathways, which use different neurotransmitter systems. It is via these pathways that extrahypothalamic structures may modify LHRH release, and consequently modulate gonadotropin secretion [2]. The study of the neurons which synthesize LHRH is made difficult by the peculiar anatomy of the LHRH system, which is composed only of a few hundreds of neurons scattered in the hypothalamic area.

A promising tool for the investigation of these interactions became available recently, when an immortalized line of LHRH-producing neurons (the GT1 cell line) was developed [16]. The three subclones of the GT1 cells so far obtained (GT1-1, GT1-3, GT1-7) [16] show the phenotypic characteristics of neuronal cells, and synthesize and secrete abundant amounts of LHRH [17]. If one assumes that these cell lines reflect the biological characteristics of natural LHRH-synthesizing neurons, they may be used to extend our knowledge on the physiology of LHRH secreting cells. Recently, it has been shown that the GT1 cells possess the receptors for several families of neurotransmitters known to modify LHRH secretion, like those for norepinephrine, dopamine, GABA, excitatory amino acids, endothelin, etc. (see [18] for a review).

It was also felt of interest to verify whether opioid receptors might be present on GT1 cells. To this purpose, the possible binding of the non-selective opioid ligand diprenorphine (DIP) on GT1-1 cells was first analyzed, using both membrane preparations and intact cells. Since it is known that opioids exert their effects through the interaction with at least three subclasses of specific binding sites (named, respectively, μ, δ and κ) [3], it was subsequently attempted to clarify whether one or more subclasses of opioid receptors might be present on this LHRH-producing cell line.

The results described in the present study provide the first evidence showing that LHRH-producing GT1-1 cells express opioid receptors. The non-selective opioid antagonist diprenorphine (DIP) binds with high affinity (0.2 nM) both to crude cell membrane preparations as well as to intact GT1-1 cells (data not shown). The binding affinity of DIP to GT1-1 cells resulted to be similar to that reported for this opiate when tested on membrane preparations obtained from either the rat brain or neuroblastoma cells [19, 20].

Selective displacement experiments performed with selective ligands for δ-, κ- and μ-receptors have revealed the presence of binding sites of the delta type only. Indeed, in the membrane preparation studies, the selective ligand for δ-opioid receptors DPDPE strongly inhibits ($>90\%$ at 1 μM dose) the binding of DIP, which, on the contrary, is not affected by selective ligands for the μ- and κ-opioid binding sites (Fig. 5).

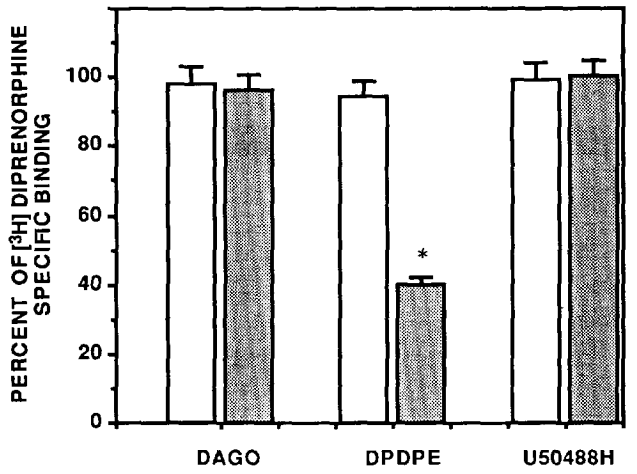

Fig. 5. Selective inhibition of the specific binding of [^{3}H]diprenorphine (0.5 nM) by μ (DAGO), δ (DPDPE) and κ (U50488H)-selective ligands. [^{3}H]diprenorphine was incubated with 1 nM (open columns) or 100 nM (shaded columns) concentration of the specific opioid agonists. Values are means ± SEM of triplicate determinations obtained from two independent experiments. *$P < 0.05$ vs [^{3}H]diprenorphine specific binding in absence of opioid agonists.

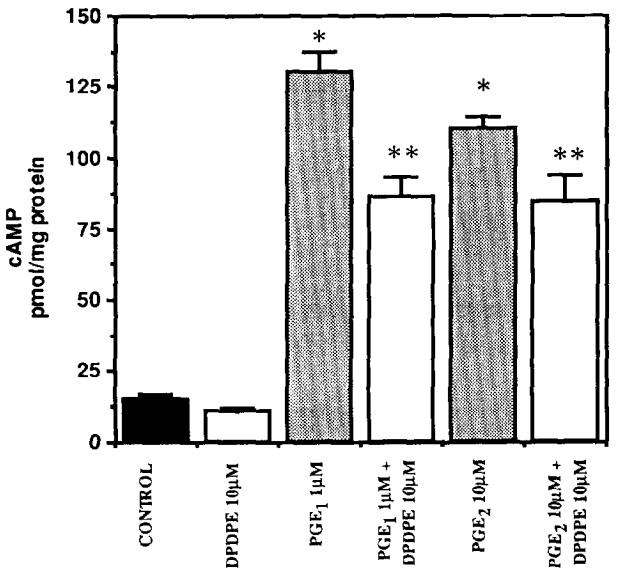

Fig. 6. Inhibition of PGE_1- and PGE_2-stimulated cAMP accumulation in GT1-1 cells by the opioid δ-agonist DPDPE. Cells were incubated for 30 min with IBMX (0.5 mM) in serum-free medium; PGE1 (1 μM) or PGE_2 (10 μM), alone or with DPDPE (10 μM), were then added for the last 15 min of culture. Values are means ± SEM of quadruplicate determinations obtained from two independent experiments. *$P < 0.05$ vs control; **$P < 0.05$ vs prostaglandin treated cells.

In order to investigate whether the δ-opioid receptors identified on GT1-1 cells have some functional significance, the ability of the δ-agonist DPDPE to inhibit the stimulation of cAMP accumulation induced in GT1-1 cells by the treatment with two known activators of adenylyl cyclase as PGE_1 and PGE_2 [21] was analyzed.

The results obtained have clearly shown that both PGs are also strong stimulators of cAMP accumulation in GT1-1 cells, and that the activation of opioid δ-receptors by DPDPE leads to a significant decrease of stimulated-cAMP levels (Fig. 6). These data suggest that the δ-receptors present on GT1-1 cells have a functional role at least on intracellular cAMP activated pathways. Furthermore, experiments in progress in this laboratory suggest that δ-agonists inhibit the release of LHRH from GT1-1 cells induced by forskolin (R. Maggi, in preparation).

The presence of δ-opioid binding sites on the subclone of GT1 cells used in the present experiments is relevant, especially in the light of the possible role of these receptors in the control of LHRH release and expression. As mentioned in the Introduction, a large body of evidence implicates opioid peptides in the central mechanisms controlling gonadotropin and LHRH secretion, mostly acting through μ-receptors [4]. The theory prevailing so far proposes that opioid neurons have synaptic contacts with intermediate neurons, which in turn influence LHRH neurons via the local release of their respective neurotransmitters [2]. Data have been collected showing that, in the brain, opioid agonists may modulate the synthesis, release and turnover of norepinephrine and dopamine (see [2] for references). If one assumes that GT1 cells represent a good model for the study of LHRH secreting neurons, the obvious conclusion from the data reported here is that endogenous opioid peptides may also influence LHRH secretion by acting directly on LHRH secreting neurons. A second conclusion would be that this effect of the opioids is mediated by the δ-subclass of receptors.

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