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Sex Steroid Influence on Cannabinoid CB_1 Receptor mRNA and Endocannabinoid Levels in the Anterior Pituitary Gland

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Recent studies have demonstrated the occurrence of endocannabinoid synthesis and of gene expression and immunoreactivity for the cannabinoid CB_1 receptor in the anterior pituitary gland. Since the activity of this gland is under the influence of circulating sex steroids, the present study was designed to elucidate whether expression of the CB_1 receptor gene in the anterior pituitary gland is also under the influence of these steroids. To this aim, we first examined the possible changes in the levels of CB_1 receptor-mRNA transcripts in the anterior pituitary gland of intact male rats and normal cycling female rats at the different stages of the ovarian cycle. We observed that males had higher levels of CB_1 receptor-mRNA transcripts than females. In addition, these transcripts fluctuated in females during the different phases of the ovarian cycle, with the highest values observed on the second day of diestrus and the lowest on estrus. In these animals, we also measured the content of endocannabinoids in the anterior pituitary gland and the hypothalamus. We observed that females had higher contents of anandamide than males in both cases. The content of anandamide in females also fluctuated during the ovarian cycle in both the anterior pituitary gland and the hypothalamus. The highest values in the anterior pituitary gland were found in the estrus and the lowest on the first day of diestrus and proestrus, whereas the inverse tendency was found in the hypothalamus. No changes were observed in the other major endocannabinoid, 2-arachidonoyl-glycerol, between males and females and during the ovarian cycle. To further explore the potential influence of circulating sex steroids on CB_1 receptor gene expression in the anterior pituitary gland, as a second objective, we examined the possible changes in the amount of transcripts for this receptor in gonadectomized and sex steroid-replaced gonadectomized rats of both sexes. We observed that orchidectomy (ORCHX) in males reduced CB_1 receptor-mRNA levels, whereas replacement with dihydrotestosterone also maintained low levels of this messenger. In females, estradiol-replaced ovariectomized (OVX) rats exhibited significantly lower CB_1 receptor-mRNA levels than OVX animals that had not been replaced with this estrogen. In this experiment, we also examined if the previously reported response of the CB_1 receptor gene in the anterior pituitary lobe to chronic administration of Δ^9-tetrahydrocannabinol (Δ^9-THC) is under sex steroid influence. We observed that chronic Δ^9-THC treatment decreased CB_1 receptor-mRNA levels in intact and ORCHX males, but not in dihydrotestosterone-replaced ORCHX males. In females, Δ^9-THC treatment produced no effect in both OVX- and estradiol-replaced OVX rats. In summary, these data collectively support that expression of the CB_1 receptor gene in the anterior pituitary gland is regulated by sex steroids in both males and females. Furthermore, gonadal steroids appear to affect the response of this gene to chronic cannabinoid administration. We have also observed that anandamide contents in the anterior pituitary gland and the hypothalamus might be controlled by circulating sex steroids. The functional implications of these data are discussed.

Recent evidence has led to revise the notion that the well-known and extensively-reported effects of synthetic and plant-derived cannabinoids (1–7) and, more recently, of endocannabinoids (7–11) on the release of anterior pituitary hormones are exclusively mediated by cannabinoid CB_1 receptors located in hypothalamic neurons that participate in the neuroendocrine control (7, 12–20). Thus, we have recently suggested that CB_1 receptors located in anterior pituitary cells are also

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involved, at least in part, in the neuroendocrine effects of cannabinoids (21–23). This suggestion was based on the recent demonstration of cannabinoid CB_1 receptor gene expression (21) and immunoreactivity (22), as well as of endocannabinoid synthesis (21), in the anterior pituitary gland. This was consistent with the previous observation by Herkenham and co-workers (24, 25) of the existence of small but specific binding for synthetic cannabinoid ligands in the pituitary, although these authors had laid little emphasis on this fact due to the very sparse distribution of specific binding found.

Our demonstration of both gene expression (21) and immunoreactivity (22) for the CB_1 receptor accounted for the previously-reported activity of Δ^9-tetrahydrocannabinol (Δ^9-THC) at the anterior pituitary level (26), although this effect was seemingly related to the activation of mechanisms other than cannabinoid receptors, such as estrogen-related pathways. Our results also explained why endocannabinoids were able to directly affect the *in vitro* release of some anterior pituitary hormones (23), in particular of prolactin. The effect on prolactin was reversed by SR141716A, a specific antagonist for the CB_1 receptor subtype, suggesting a role of this receptor in the pituitary on the control of the release of this pituitary hormone. Using double-labeling analysis, we have observed immunoreactivity for CB_1 receptor and prolactin in the same cells, although anterior pituitary cells other than lactotrophs (mainly gonadotrophs) were also stained with the CB_1 receptor antibody (22). We have also observed that incubation of anterior pituitary explants with Δ^9-THC resulted in increased ACTH secretion and this increase was blocked by SR141716A (González, Manzanares, Ambrosio, Oliva, Ramos, and Fernández-Ruiz, unpublished results). A similar increase in ACTH release has been also observed with anandamide using dispersed anterior pituitary cells (23), although there was no blockade by CB_1 receptor antagonists. It is possible that corticotroph cells could also contain a cannabinoid receptor subtype other than the CB_1, since we did not observe a good correlation between CB_1 receptor and ACTH immunoreactivities (22).

The present study was designed to further explore the physiological significance of gene expression (21) and immunoreactivity (22) for the CB_1 receptor and of endocannabinoid synthesis (21) in the anterior pituitary gland. It is well-demonstrated that the activity of most of the anterior pituitary cells is under the influence of circulating sex steroids in both males and females [for review, see (27)]. Thus, we wanted to examine whether the expression of CB_1 receptor gene in the anterior pituitary gland is under sex steroid-dependent regulation. To this aim, in a first experiment, we analyzed the levels of transcripts for this receptor in intact males and normal cycling females at the different stages of ovarian cycle. In this experiment, we also measured the contents of anandamide and 2-arachidonoyl-glycerol (2-AG), the two major endocannabinoids, in the anterior pituitary gland and the hypothalamus of the same animal groups. In a second experiment, we examined whether gonadectomy and sex steroid replacement in both sexes resulted in changes in CB_1 receptor-mRNA levels in the anterior pituitary gland. In this second experiment, we also examined whether the recently demonstrated down-regulatory response of CB_1 receptor gene in the anterior pituitary lobe after the chronic administration of Δ^9-THC (21) was also under sex steroid influence.

MATERIALS AND METHODS

Animals, treatments, and sampling. Male and female rats of the Sprague–Dawley strain were housed from birth in a room with a controlled photoperiod (lights on: 8 AM–8 PM) and temperature (23 ± 1°C). They had free access to standard food (Panlab) and water. At adult age (>8 weeks of life; 250 ± 25 g), animals were used for two different experiments (all experiments were conducted according to European Animal Care Guides). In experiment I, females were subjected to control of ovarian cycle (daily vaginal smears were taken around 10:00 h) and only those animals exhibiting at least 3 or more consecutive 4-day cycles were used in this study. Rats were sacrificed by rapid decapitation at the four different phases of the estrous cycle (diestrus 1, diestrus 2, proestrus and estrus). Intact males were also sacrificed in a number similar to that used for each ovarian phase in females. After sacrifice, the pituitary gland and the hypothalamus were quickly and carefully removed. All this process was carried out at room temperature and for a period not longer than 1 min in each rat. Pituitaries to be used for analysis of CB_1 receptor-mRNA levels by *in situ* hybridization were rapidly frozen by immersion in a 2-methyl-butane dry ice bath. Samples were stored at −70°C until analyzed. Pituitaries or hypothalami to be used for analysis of endocannabinoid contents were pooled in a number of 10 for each individual measurement and homogenized in 5 vol of chloroform/methanol (2:1) and subjected to lipid extraction, as will be described below. In experiment II, males and females were bilaterally gonadectomized under ether anesthesia and left 7 days for recovery. Then, half of gonadectomized animals were implanted with Silastic capsules (30 mm length; 1.57 mm i.d.; 3.8 mm o.d.). In the case of females, the capsules contained 37.5 μg/ml of 17β-estradiol benzoate (Sigma Chem., Madrid, Spain) dissolved in sunflower oil. This procedure led to constant plasma estrogen levels similar to those found in the diestrus (28). In the case of males, the capsules contained crystalline dihydrotestosterone propionate (Sigma Chemicals, Madrid, Spain) (29). Dihydrotestosterone was used instead testosterone to avoid aromatization to estradiol. The other half of gonadectomized animals for each sex was implanted with empty capsules to be used as controls. Seven days after silastic capsule implantation, each experimental group was subjected to a daily administration of Δ^9-THC (5 mg/kg wt/day; ip; 1 ml/kg), kindly supplied by the National Institute on Drug Abuse (Rockville, MD), or vehicle [(1:1:18) ethanol–cremophor–saline], for a period of 7 days. In the case of males, a group of intact animals, subjected to the corresponding sham-operations, were also subjected to daily ip administration of Δ^9-THC (5 mg/kg wt) or vehicle for a period of 7 days, as used as a positive control (21). Animals were sacrificed by rapid decapitation 24 h after the last injection. After sacrifice, the pituitary gland was quickly and carefully removed and processed as in the above experiment for the analysis of CB_1 receptor-mRNA levels.

Analysis of CB_1 receptor-mRNA levels by in situ hybridization. *In situ* hybridization was carried out according to the procedure previously described by Rubino *et al.* (30). Briefly, pituitaries were cut in sections 20 μm thick in a cryostat. Sections were thaw-mounted onto

RNAse-free gelatin/chrome alum coated slides, dried briefly at 30°C and stored at −80°C until used. Pituitary sections were fixed in 4% formaldehyde for 5 min and, after rinsing twice in phosphate buffer saline, were acetylated by incubation in 0.25% acetic anhydride, prepared in 0.1 M triethanolamine/0.15 M sodium chloride (pH 8.0), for 10 min. Sections were rinsed in 0.3 M sodium chloride/0.03 M sodium citrate, pH 7.0, dehydrated and delipidated by ethanol/chloroform series. A mixture (1:1:1) of the three 48-mer oligonucleotide probes complementary to bases 4–51, 349–396, and 952–999 of the rat CB_1 receptor cDNA (Du Pont, ITISA, Madrid, Spain; the specificity of the probes used was assessed by Northern Blot analysis) was 3′-end labeled with [^{35}S]-dATP (Amersham Ibérica, Madrid, Spain) using terminal deoxynucleotidyl-transferase (Boehringer-Mannheim, Barcelona, Spain). Sections were then hybridized with [^{35}S]-labeled oligonucleotide probes (2.5×10^5 dpm *per* section), washed and exposed to X-ray film (Biomax MR, Kodak) for 10 days, and developed (D-19, Kodak) for 6 min at 20°C. The intensity of the hybridization signal was assessed by measuring the gray levels in the autoradiographic films with a computer-assisted videodensitometer (Image Quant 3.3, Molecular Dynamics). Additional pituitary sections were co-hybridized with a 100-fold excess of cold probe or with RNAse to assert the specificity of the signal.

Analysis of endogenous cannabinoid levels by GC–MS. As mentioned above, pooled pituitaries or hypothalami were homogenized in 5 vol of chloroform/methanol (2:1) and subjected to lipid extraction. Homogenates were centrifuged at 13,000*g* for 16 min (4°C), the supernatants collected and the precipitates resuspended in 5 vol of chloroform/methanol (2:1) and again centrifuged. This procedure was repeated three times more. Afterwards, the supernatants collected in the 5 consecutive centrifugations for each pool of tissues were mixed and the organic solvents evaporated in a Speed-Vac. Lyophilized samples were then stored frozen at −80°C under nitrogen atmosphere until analyzed. Lyophilized extracts were resuspended in chloroform/methanol 99:1 by vol. and 5 nmol of deuterated anandamide and 2-AG added to the solution. The two deuterated standards were synthesized from d_8 arachidonic acid and ethanolamine or glycerol as described, respectively, in Devane *et al.* (31) and Bisogno *et al.* (32). The solutions were then purified by open bed chromatography on silica as described in Fontana *et al.* (33). Fractions eluted with chloroform/methanol 9:1 by vol. (containing anandamide and 2-AG) were collected and the excess solvent evaporated with a rotating evaporator. The former fractions were further fractionated by normal phase high pressure liquid chromatography (NP-HPLC) carried out using a semi-preparative silica column (Spherisorb S5W, Phase Sep, Queensferry, CLWYD, UK) eluted with a 40-min linear gradient from 9:1 to 8:2 (by volume) of n-hexane/2-propanol (flow rate = 2 ml/min). These elution conditions allow the separation of 1(3)- and 2-acyl-glycerols (retention time of 18 and 20 min, respectively) from *N*-acylethanolamines (retention time = 26–27 min). NP-HPLC fractions from 17 to 22 min and from 24 to 28 min were pooled, the solvent evaporated in a Speed-Vac, and the components derivatized with 20 μl N-methyl-N-trimethylsilyl-trifluoroacetamide + 1% trimethylchlorosylane for 2 h at room temperature and analyzed by GC–MS carried out under conditions described previously (32) and allowing the separations of monoacylglycerols or *N*-acylethanolamines with different fatty acid chains. MS detection was carried out in the selected ion monitoring mode using m/z values of 427 and 419 (molecular ions for deuterated and undeuterated anandamide), 412 and 404 (loss of 15 mass units from deuterated and undeuterated anandamide), 530 and 522 (molecular ions for deuterated and undeuterated 2-AG), and 515 and 507 (loss of 15 mass units from deuterated and undeuterated 2-AG). The area ratios between signals of deuterated and undeuterated anandamide varied linearly with varying amounts of undeuterated anandamide (20 pmol–20 nmol). The same was applied to the area ratios between signals of deuterated and undeuterated 2-AG in the 100-pmol to 20-nmol interval. Anandamide and 2-AG levels in unknown samples were therefore calculated on the basis of their area ratios with the internal deuterated standard signal areas. Two GC–MS peaks for both deuterated and undeuterated mono-arachidonoylglycerol were found, corresponding to 2-AG and 1(3)-AG, in agreement with the previous observation that 2-AG undergoes isomerization during the purification procedure (34). Therefore, the amounts of 2-AG were calculated by adding the amounts of the two isomers. The amounts of endocannabinoids are expressed as picomoles or nanomoles per gram of wet tissue extracted.

Statistics. Data of the experiment I (CB_1 receptor-mRNA levels and endocannabinoid contents in the pituitary gland and the hypothalamus of intact males and normal cycling females) were assessed by using one-way analysis of variance followed by the Student–Newman–Keuls test, whereas data of the experiment II (CB_1 receptor-mRNA levels in the pituitary gland of Δ^9-THC-treated gonadectomized and sex steroid-replaced gonadectomized rats) were assessed by using two-way analysis of variance (Δ^9-THC treatment × sex steroid treatment) followed by the Student–Newman–Keuls test.

RESULTS

Experiment I: Levels of CB_1 Receptor-mRNA Transcripts in the Anterior Pituitary Gland and Endocannabinoids in the Anterior Pituitary Gland and the Hypothalamus of Intact Males and Normal Cycling Females

In this experiment, we first examined the levels of CB_1 receptor-mRNA transcripts in the anterior pituitary gland of intact males and normal cycling females. We observed that males had higher levels of CB_1 receptor-mRNA transcripts in the anterior pituitary gland than females (Table 1). The levels of CB_1 receptor-mRNA transcripts also fluctuated in the anterior pituitary gland of females through the different phases of the ovarian cycle, with the highest values observed in the second day of diestrus and the lowest one in the estrus (Table 1).

In these animals, we also measured the contents of endocannabinoids in the pituitary gland and the hypothalamus. We observed that females had higher contents of anandamide than males in both cases (Fig. 1). The contents of anandamide also fluctuated during the ovarian cycle in the pituitary gland of females (Fig. 1). The highest values were found in the estrus and the lowest in the first day of diestrus and the proestrus (Fig. 1). By contrast, the pattern followed by anandamide contents in the hypothalamus through the ovarian cycle exhibited an interesting inverse correlation with that in the pituitary gland ($r = -0.7786$) (see Fig. 1), although the differences in the hypothalamus did not reach statistical significance. On the other hand, males seemed to exhibit higher 2-AG contents in the pituitary gland, but lower in the hypothalamus, than females, although 2-AG data did not reach statistical significance in any cases, using analysis of variance, for both the differences between males and females, and the fluctuations during the ovarian cycle in females (Table 1).

TABLE 1

CB_1 Receptor mRNA Levels, Measured by *in Situ* Hybridization, in the Anterior Pituitary Gland and Concentrations of 2-Arachidonoyl-glycerol, Measured by Gas Chromatography–Mass Spectrometry, in the Anterior Pituitary Gland and the Hypothalamus of Adult Intact Male and Normal Cycling Female Rats

Groups	CB_1 receptor mRNA levels (arbitrary units of optical density)	2-Arachidonoyl-glycerol (nmol/g tissue)	
		Hypothalamus	Anterior pituitary
Females			
Diestrus (1st day)	6.1 ± 2.6^a	11.8 ± 1.7	8.8 ± 0.3
Diestrus (2nd day)	15.2 ± 3.8^b	9.9 ± 0.5	5.5 ± 1.4
Proestrus	5.7 ± 2.0^a	10.2 ± 1.2	7.8 ± 1.5
Estrus	3.0 ± 0.9^a	9.1 ± 0.9	7.0 ± 1.6
Males	21.3 ± 2.2^b	6.0 ± 2.0	10.8 ± 2.4
Statistics	$F(4,23) = 8.705$, $P < 0.005$	$F(4,13) = 2.256$, $P = 0.143$	$F(4,13) = 2.21$, ns

Note. Details in the text. Values are means ± SEM ($n = 5–7$ per group for the analysis of CB_1 receptor mRNA levels and $n = 3$ per group for the analysis of 2-arachidonoyl-glycerol contents). Statistical differences were assessed by one-way analysis of variance followed by Student–Newman–Keuls test. Values with common superscript letters are not statistically different.

Experiment II: Response of CB_1 Receptor Gene in the Anterior Pituitary Gland of Gonadectomized and Sex Steroid-Replaced Rats to a Chronic Δ^9-THC Administration

In this experiment, we first examined whether CB_1 receptor-mRNA levels measured in the anterior pituitary gland were altered after gonadectomy and sex steroid-replacement in both sexes. We observed that orchidectomy (ORCHX) in males reduced CB_1 receptor-mRNA levels, and replacement with dihydrotestosterone did not change significantly the effect of ORCHX (Table 2). In females, estradiol-replaced ovariectomized (OVX) rats exhibited significantly lower CB_1 receptor-mRNA levels in the anterior pituitary gland than OVX animals that had not been replaced with this estrogen (Table 2).

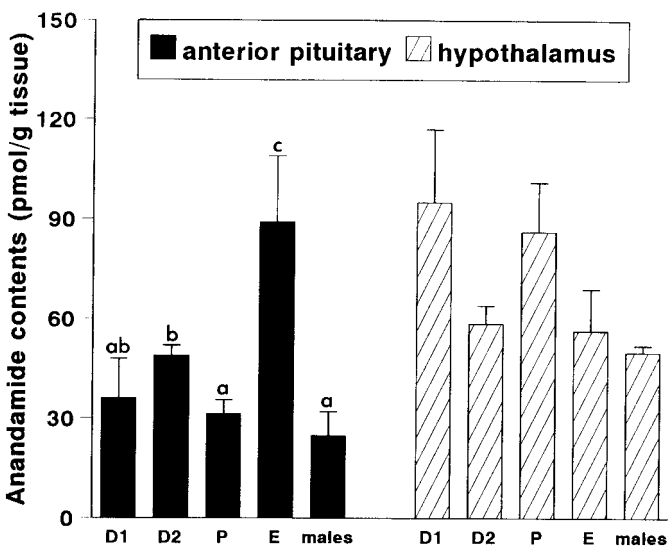

FIG. 1. Concentrations of anandamide, measured by gas chromatography–mass spectrometry, in the anterior pituitary gland and the hypothalamus of adult intact male and normal cycling female rats. Details in the text. Values are means ± SEM of three determinations *per* group. Statistical differences were assessed by one-way analysis of variance (anterior pituitary gland: $F(4,12) = 3.954$, $P < 0.05$; hypothalamus: $F(4,14) = 1.951$, $P = 0.178$) followed by Student–Newman–Keuls test. Values with common letters are not statistically different.

In this experiment, we also examined if the previously reported down-regulatory response of CB_1 receptor gene in the anterior pituitary lobe to the chronic administration of Δ^9-THC is under sex steroid influence. We observed that chronic Δ^9-THC treatment decreased CB_1 receptor-mRNA levels in the anterior pituitary gland of intact and ORCHX males, but not in dihydrotestosterone-replaced ORCHX males (Table 2). In females, Δ^9-THC treatment produced no effect in both OVX and estradiol-replaced OVX rats (Table 2).

DISCUSSION

In recent studies, we have demonstrated the presence of mRNA transcripts (21) and immunoreactivity (22) for the CB_1 receptor in the anterior pituitary gland. We have also provided evidence, by using double-labeling staining, that cells that contain CB_1 receptor in the anterior lobe of the pituitary gland are mostly PRL-secreting cells, although immunoreactivity for the CB_1 receptor was also found in gonadotrophs (22). In addition, although previous studies demonstrated that classic plant-derived cannabinoids, such as Δ^9-THC, were unable to alter PRL secretion from incubated pituitary glands (20), we have preliminary evidence that anandamide affects PRL secretion *in vitro* from dispersed anterior pituitary cells and this effect is blocked by CB_1 receptor antagonists (23). All this supports the existence of functional receptors acting directly at the anterior pituitary level in the control of hormone secretion, in particular of PRL secretion.

TABLE 2

Effects of Chronic (7 Days) Administration of Δ^9-Tetrahydrocannabinol (Δ^9-THC) on CB_1 Receptor mRNA Levels (Arbitrary Units of Optical Density), Measured by *in Situ* Hybridization, in the Anterior Pituitary Gland of Gonadectomized and Sex Steroid-Replaced Gonadectomized Male and Female Rats

Groups	+Vehicle	+Δ^9-THC	Statistics (two-way ANOVA)
Females			
OVX	22.2 ± 3.0	27.4 ± 5.9	Δ^9-THS treatment: $F(1,27) = 0.29$, ns
OVX + estradiol	9.4 ± 2.7†	7.5 ± 1.2††	Steroid treatment: $F(1,27) = 22.91$, $P < 0.0005$
			Two-way interaction: $F(1,27) = 1.10$, ns
Males			
Intact	16.9 ± 1.3	10.1 ± 1.2**	Δ^9-THC treatment: $F(1,39) = 10.23$, $P < 0.005$
ORCHX	12.6 ± 1.3§	8.9 ± 0.8*	Steroid treatment: $F(2,39) = 4.17$, $P < 0.05$
ORCHX + DHT	9.8 ± 1.3§§	11.1 ± 1.0	Two-way interaction: $F(2,39) = 6.03$, $P < 0.05$

Note. Details in the text. Values are means ± SEM of seven to nine determinations per group. Statistical differences were assessed for each sex by two-way analysis of variance (Δ^9-THC treatment × sex steroid treatment) followed by Student–Newman–Keuls test (*$P < 0.05$, **$P < 0.005$ vs. the corresponding vehicle-treated group; †$P < 0.005$, ††$P < 0.0005$ vs the corresponding gonadectomized group; §$P < 0.05$, §§$P < 0.05$ vs the intact group in males).

The present study provides further information on these pituitary CB_1 receptors, since we have demonstrated that the expression of CB_1 receptor gene in the anterior pituitary gland is subjected to sex steroid-dependent influence, much in the same way as the activity of anterior pituitary cells is [for review, see (27)]. Thus, CB_1 receptor mRNA levels exhibited differences between males and females, and fluctuated during the ovarian cycle in females. This is concordant with the observation that the effects of exogenous cannabinoids are sex-dependent and also vary depending on the ovarian phase in females [for review, see (14–17)]. For instance, it has been previously shown that males are more sensitive than females to cannabinoid-induced changes in pituitary hormone secretion (15, 16). The present study provides evidence that the amounts of CB_1 receptor mRNA transcripts in the anterior pituitary gland of males are higher than those in females. Moreover, in females we observed a rise in the amounts of CB_1 receptor mRNA transcripts in the anterior pituitary gland during the second day of the diestrus. These changes are fully concordant with our previous data on CB_1 receptor binding in the medial basal hypothalamus (35), where also higher binding in males than females and fluctuations during the ovarian cycle with a peak in the diestrus were found (35).

The influence of circulating sex steroids in the basal expression of CB_1 receptor gene in the anterior pituitary gland is also supported by results obtained in the present study using gonadectomized and sex steroid-replaced animals. In males, castration reduced CB_1 receptor mRNA levels in the anterior lobe of the pituitary gland, suggesting a possible stimulatory influence of androgens in the expression of this gene. Replacement with dihydrotestosterone did not recover the levels found in intact males, thus suggesting that CB_1 receptor gene might be under the stimulatory influence of testosterone only following its aromatization to estradiol. Further studies using replacement with testosterone and aromatase inhibitors will be required to demonstrate this hypothesis. In females, castration apparently increased CB_1 receptor gene expression in the anterior pituitary gland, whereas replacement with estradiol markedly reduced the amount of transcripts to the levels found in the diestrous phase. This finding suggests that estradiol might negatively regulate the expression of CB_1 receptor gene in the anterior pituitary gland of females. Assuming that CB_1 receptors located in this pituitary lobe are involved in the inhibitory control of, among others, PRL secretion, as suggested by recent *in vitro* (23) and double-labeling (22) studies, the decrease in the levels of CB_1 receptor-mRNA observed in the anterior pituitary gland of OVX rats replaced with estradiol would be consistent with the increase in plasma PRL levels observed previously with his hormone (17) under conditions similar to those used in the present study.

In a previous study (21), we demonstrated the presence of endogenous ligands for CB_1 receptors in the anterior pituitary gland as well as in the hypothalamus. We observed for the first time that 2-AG and anandamide are present in the anterior pituitary gland. Furthermore, the presence of its lipid precursor, *N*-arachidonoyl-phosphatidylethanolamine, suggests that anandamide could be synthesized in the pituitary (21). This finding supports the involvement of endocannabinoids in the control of anterior pituitary hormone secretion, not only at the hypothalamic level, as previously suggested (7, 12–20), but also through their action at CB_1 receptors located in the pituitary. In the present study, we have again detected measurable amounts of 2-AG and anandamide in the pituitary gland, as well as in the hypothalamus. These amounts do not necessarily reflect the amounts that are actually released from afferent pituitary neurons and pituitary cells. For example, it is known that under the same

experimental conditions, about 50% of anandamide, but only 20% of 2-AG, biosynthesized de novo is released outside neurons (32). Furthermore, anandamide and 2-AG are selectively released from striatum and hippocampus, respectively (34, 36). So, it is possible that only a minor part of the 2-AG found by us in the pituitary acts as an extracellular mediator.

In our experiment, we have observed that pituitary and hypothalamic contents of anandamide are also under a possible sex steroid influence. Thus, in contrast with what we observed here for CB_1 receptor-mRNA levels, the contents of anandamide in the pituitary gland and the hypothalamus were higher in females than males, thus indicating sex-dependent differences in the ligand/receptor ratios. In addition, anandamide contents in the anterior pituitary gland peaked in the estrous phase, but exhibited lower levels in the second day of diestrus, in clear difference with the fluctuations of CB_1 receptor-mRNA transcripts. On the other hand, there was a possible correspondence between the contents of anandamide in the hypothalamus and the amounts of CB_1 receptor-mRNA transcripts in the anterior pituitary gland through the ovarian cycle. In fact, although the values found in the two days of the diestrus were clearly different for both parameters, the lowest values were always measured in the estrus, whereas intermediate values were found in the proestrus. This may indicate that anandamide acting on pituitary CB_1 receptors has a hypothalamic rather than a pituitary origin. However, this might change during some phases of the ovarian cycle since the correspondence between the two parameters was not always direct (i.e., the diestrous phase). Anyway, this hypothesis will require further investigation.

It is worthwhile mentioning that there also was a good inverse correlation between the patterns followed by anandamide contents in the anterior pituitary gland and in the hypothalamus during the ovarian cycle. This might suggest the existence of a feed-back control between the two regions and/or that the regulation by sex steroids of anandamidergic activity in the hypothalamus is the opposite of that in the pituitary gland, which might have important physiological implications. It is also important to note that the levels of the other endocannabinoid analyzed, 2-AG, did not vary significantly during the ovarian cycle or between males and females in both the anterior pituitary gland and the hypothalamus, as also observed in previous analytical studies carried out in the rat brain (37). The finding of variations in anandamide, but not 2-AG, contents during the ovarian cycle may suggest that, in the pituitary, anandamide rather than 2-AG plays a physiological role. It appears, therefore, that the levels of CB_1 receptors and endogenous cannabinoid ligands are subjected to a complex and differential regulation by sex steroids.

Finally, the last goal of this study was to assess whether the recently-reported response (inhibition followed by stimulation) of CB_1 receptor-mRNA transcripts in the anterior pituitary gland to the chronic exposure to synthetic or plant-derived cannabinoids (21), is also under sex steroid influence. Thus, we analyzed the levels of transcripts after a 7-day period of daily Δ^9-THC treatment to gonadectomized and sex steroid-replaced animals. Results demonstrated that chronic Δ^9-THC treatment had no effect on the amount of CB_1 receptor transcripts in both OVX and estradiol-replaced OVX rats. This is concordant with previous observations that females are less vulnerable to (or better protected from) the effects of chronic cannabinoid exposure (for review, see (38)). On the contrary, in males, chronic Δ^9-THC treatment produced a marked reduction in CB_1 receptor-mRNA transcripts in intact and ORCHX animals, as observed previously (21), but was ineffective in dihydrotestosterone-replaced ORCHX animals, suggesting that this response might be also under androgen influence.

In summary, these data collectively support the conclusion that the expression of CB_1 receptor gene in the anterior pituitary gland is under the control of sex steroids in both males and females and this control also affects the response of this gene to chronic activation of CB_1 receptors with a specific agonist. We have also observed that endocannabinoid contents in the anterior pituitary gland, as well as in the hypothalamus, might be also controlled by circulating sex steroids. The functional implications of these data are discussed.

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REFERENCES

1. Murphy, L., Steger, R. W., Smith, M. S., and Bartke, A. (1990) *Neuroendocrinology* **52,** 316–321.
2. Murphy, L. L., Steger, R. W., and Bartke, A. (1990) *in* Biochemistry and Physiology of Substance Abuse (Watson, R. R., Ed.), Vol. 2, pp. 73–93, CRC Press, Boca Raton, FL.
3. Rodríguez de Fonseca, F., Fernández-Ruiz, J. J., Murphy, L. L., Cebeira, M., Steger, R. W., Bartke, A., and Ramos, J. A. (1992) *Pharmacol. Biochem. Behav.* **42,** 269–275.
4. Jackson, A. L., and Murphy, L. (1997) *Neuroendocrinology* **65,** 446–452.
5. Rodríguez de Fonseca, F., Fernández-Ruiz, J. J., Murphy, L. L., Eldridge, J. C., Steger, R. W., and Bartke, A. (1991) *Pharmacol. Biochem. Behav.* **40,** 593–598.
6. Kubena, R. K., Perhack, J. C., and Barry, H. (1971) *Eur. J. Pharmacol.* **14,** 89–92.

7. Fernández-Ruiz, J. J., Muñoz, R. M., Romero, J., Villanua, M. A., Makriyannis, A., and Ramos, J. A. (1997) *Biochem. Pharmacol.* **53,** 1919–1927.
8. Romero, J., García-Gil, L., Ramos, J. A., and Fernández-Ruiz, J. J. (1994) *Neuroendocr. Lett.* **16,** 159–164.
9. Wenger, T., Fragakis, G., Probonas, K., Toth, B. E., and Yiannakakis, N. (1994) *Neuroendocr. Lett.* **16,** 295–303.
10. Wenger, T, Jamali, K. A., Juaneda, C., Leonardelli, J., and Ttamu, G. (1997) *Biochem. Biophys. Res. Commun.* **237,** 724–728.
11. Weidenfeld, J., Feldman, S., and Mechoulam, R. (1994) *Neuroendocrinology* **59,** 110–112.
12. Romero, J., Wenger, T., De Miguel, R., Ramos, J. A., and Fernández-Ruiz, J. J. (1998) *Life Sci.* **63,** 351–356.
13. Kramer, J., and Ben-David, M. (1978) *Endocrinology* **103,** 452–458.
14. Fernández-Ruiz, J. J., Navarro, M., Hernández, M. L., Vaticón, D., and Ramos, J. A. (1992) *Neuroendocr. Lett.* **14,** 349–355.
15. Bonnin, A., Ramos, J. A., Rodríguez de Fonseca, F., Cebeira, M., and Fernández-Ruiz, J. J. (1993) *Neuroendocrinology* **58,** 280–286.
16. Bonnin, A., De Miguel, R., Fernández-Ruiz, J. J., Cebeira, M., and Ramos, J. A. (1994) *Biochem. Pharmacol.* **48,** 1387–1392.
17. Bonnin, A., Fernández-Ruiz, J. J., Cebeira, M., and Ramos, J. A. (1994) *Neuroendocr. Lett.* **16,** 305–314.
18. Murphy, L. L., Chandrashekar, V., and Bartke, A. (1994) *Neuroendocr. Lett.* **16,** 1–7.
19. De Miguel, R., Romero, J., Muñoz, R. M., García-Gil, L., González, S., Villanua, M. A., Makriyannis, A., Ramos, J. A., and Fernández-Ruiz, J. J. (1998) *Biochem. Pharmacol.* **56,** 1331–1338.
20. Hughes, C. L., Everett, J. W., and Tyrey, L. (1981) *Endocrinology* **109,** 876–880.
21. González, S., Manzanares, J., Berrendero, F., Wenger, T., Corchero, J., Bisogno, T., Romero, J., Fuentes, J. A., Di Marzo, V., Ramos, J. A., and Fernández-Ruiz, J. J. (1999) *Neuroendocrinology* **70,** 137–145.
22. Wenger, T., Fernández-Ruiz, J. J., and Ramos, J. A. (1999) *J. Neuroendocr.* **11,** 873–878.
23. Wenger, T., Jamali, K. A., Juaneda, C., Bacsy, E., and Tramu, G. (2000) *Addict. Biol.* **5,** 59–64.
24. Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., De Costa, B. R., and Rice, K. C. (1991) *J. Neurosci.* **11,** 563–583.
25. Lynn, A. B., and Herkenham, M. (1994) *J. Pharmacol. Exp. Ther.* **268,** 1612–1623.
26. Murphy, L. L., Newton, S. C., Dhali, J., and Chavez, D. (1991) *Pharmacol. Biochem. Behav.* **40,** 603–608.
27. Genazzani, A. R., Lucchesi, A., Stomati, M., Catarsi, S., Genazzani, A. D., Criscuolo, M., and Petraglia, F. (1997) *Eur. J. Contracept. Reprod. Health Care* **2,** 63–69.
28. Wise, P. M., Camp-Grossman, P., and Barraclough, C. A. (1981) *Biol. Rep.* **24,** 820–830.
29. Kalra, P. S., and Kalra, S. P. (1982) *Endocrinology* **111,** 24–29.
30. Rubino, T., Massi, P., Patrini, G., Venier, I., Giagnoni, G., and Parolaro, D. (1994) *Neuroreport* **5,** 2493–2496.
31. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258,** 1946–1949.
32. Bisogno, T., Sepe, N., Melck, D., Maurelli, S., De Petrocellis, L., and Di Marzo, V. (1997) *Biochem. J.* **322,** 671–677.
33. Fontana, A., Di Marzo, V., Cadas, H., and Piomelli, D. (1995) *Prostagl. Leukotr. Essent. Fatty Acids* **53,** 301–308.
34. Stella, N., Schweitzer, P., and Piomelli, D. (1997) *Nature* **388,** 773–778.
35. Rodríguez de Fonseca, F., Cebeira, M., Ramos, J. A., Martin, M., and Fernández-Ruiz, J. J. (1994) *Life Sci.* **54,** 159–170.
36. Giuffrida, A., Parsons, L. H., Kerr, T. M., Rodríguez, F., Navarro, M., and Piomelli, D. (1999) *Nat. Neurosci.* **2,** 358–363.
37. Bisogno, T., Berrendero, F., Ambrosino, G., Cebeira, M., Ramos, J. A., Fernández-Ruiz, J. J., and Di Marzo, V. (1999) *Biochem. Biophys. Res. Commun.* **256,** 377–380.
38. Wenger, T., Croix, D., Tramu, G., and Leonardelli, J. (1992) *in* Marijuana/Cannabinoids. Neurobiology and Neurophysiology (Murphy, L. L., and Bartke, A., Eds.), pp. 539–560, CRC Press, Boca Raton, FL.