Biochemical Pharmacology, Vol. 56, pp. 1331–1338, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S006-2952(98)00185-3

Effects of Cannabinoids on Prolactin and Gonadotrophin Secretion: Involvement of Changes in Hypothalamic γ-Aminobutyric Acid (GABA) Inputs

Rosario de Miguel, Julián Romero,* Raúl M. Muñoz,† Lucía García-Gil,* Sara González,* Maria A. Villanua,† Alexandros Makriyannis,‡ José A. Ramos* and J. Javier Fernández-Ruiz§*

Departments of *Biochemistry and †Human Physiology, Faculty of Medicine, Complutense University, 28040-Madrid, Spain; and ‡Department of Pharmaceutical Sciences and Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, U.S.A.

ABSTRACT. CB_1 cannabinoid receptors are located in hypothalamic nuclei and their activation alters several hypothalamic neurotransmitters resulting in, among other things, decreased prolactin (PRL) and luteinizing hormone (LH) secretion from the anterior pituitary gland. In the present study, we addressed two related objectives to further explore this complex regulation. First, we examined whether changes in γ-aminobutyric acid (GABA) and/or dopamine (DA) inputs in the medial basal hypothalamus might occur in parallel to the effects resulting from the activation of CB_1 receptors on PRL and gonadotrophin secretion in male rats. Thus, the acute administration of (-)-Δ^9-tetrahydrocannnabinol (Δ^9-THC) produced, as expected, a marked decrease in plasma PRL and LH levels, with no changes in follicle-stimulating hormone (FSH) levels. This was paralleled by an increase in the contents of GABA, but not of DA, in the medial basal hypothalamus and, to a lesser extent, in the anterior pituitary gland. The co-administration of Δ^9-THC and SR141716, a specific antagonist for CB_1 receptors, attenuated both PRL and LH decrease and GABA increase, thus asserting the involvement of the activation of CB_1 receptors in these effects. As a second objective, we tested whether the prolonged activation of these receptors might induce tolerance with regard to the decrease in PRL and LH release, and whether this potential tolerance might be related to changes in CB_1-receptor binding and/or mRNA expression. The chronic administration of R-methanandamide (AM356), a more stable analog of anandamide, the putative endogenous cannabinoid ligand, produced a marked decrease in plasma PRL and LH levels, with no changes in FSH. The decreases were of similar magnitude to those caused by a single injection of this cannabimimetic ligand, thus suggesting the absence of tolerance. In parallel, the analysis of CB_1-receptor binding and mRNA expression in several hypothalamic structures proved that the acute or chronic administration of AM356 did not affect either the binding or the synthesis of these receptors. In summary, the activation of CB_1 receptors in hypothalamic nuclei produced the expected decrease in PRL and LH secretion, an effect which might be related to an increase in GABAergic activity in the hypothalamus-anterior pituitary axis. The prolonged activation of these receptors for five days did not elicit tolerance in terms of an attenuation in the magnitude of the decrease in PRL and LH, and, accordingly, did not alter CB_1-receptor binding and mRNA levels in the hypothalamic nuclei examined. BIOCHEM PHARMACOL **56**;10:1331–1338, 1998.

KEY WORDS. prolactin; gonadotrophin; Δ^9-tetrahydrocannabinol; methanandamide; CB_1-cannabinoid receptors; GABA

The administration of Δ^9-THC,‖ the main psychoactive principle of cannabis sativa derivatives, to rodents decreases PRL and gonadotrophin release from the anterior pituitary gland at both adult [1–3] and immature ages [4–6]. Moreover, we [7, 8] and others [9, 10] have recently demonstrated that anandamide, a cannabimimetic compound that has been proposed as the endogenous ligand for the cannabinoid receptor [11], is also able to inhibit PRL secretion. The site of these inhibitory effects seems to be more the hypothalamic structures related to the neuroendocrine control of the anterior pituitary secretion rather than the anterior pituitary gland itself. Indeed, Δ^9-THC did not inhibit PRL secretion in vitro or from ectopic pituitaries [12], although a certain activity of Δ^9-THC at the anterior pituitary level has recently been suggested [13]. Accordingly, CB_1-cannabinoid receptors seem to be present, although in a small density, in hypothalamic nuclei [14, 15], but are apparently absent from the anterior pituitary gland

§ Corresponding author: Dr. J. J. Fernández-Ruiz, Department of Biochemistry, Faculty of Medicine, Complutense University, 28040-Madrid, Spain. Tel. 34-91-3941450; FAX 34-91-3941691; E-mail: jjfr@eucmax.sim.ucm.es.

‖ *Abbreviations*: AM356, R-methanandamide; DA, dopamine; FSH, follicle-stimulating hormone; GABA, γ-aminobutyric acid; LH, luteinizing hormone; PRL, prolactin; and Δ^9-THC, (-)-Δ^9-tetrahydrocannnabinol.

Received 12 January 1998; accepted 22 April 1998.

[16], clearly supporting the notion that the effects of cannabimimetics are mediated through changes in the activity of certain hypothalamic neurotransmitters. Recently, because the blockade of these receptors with a specific antagonist abolished the reduction in plasma PRL levels following Δ^9-THC administration [8], we have demonstrated the involvement of CB_1 receptors, which are located in the neuroendocrine hypothalamus, in the inhibitory effect of cannabinoids on PRL secretion. However, some studies performed in our laboratory have demonstrated that Δ^9-THC is also able to stimulate PRL secretion [8, 17], although this effect appears to be mediated through mechanism(s) other than the activation of CB_1 receptors and was not observed for gonadotrophin release [8].

Still a matter of controversy are the specific hypothalamic neurotransmitter(s) that are primarily involved in the effects of cannabinoids on the secretion of the different anterior pituitary hormones and, hence, the phenotypy of the neurons where CB_1 receptors in the hypothalamus could be located. Previous studies have reported that the administration of cannabimimetics was able to alter the activity of serotoninergic [18] and especially dopaminergic [3, 6, 17, 19–21] neurons, which are involved, directly or indirectly, in the control of neuroendocrine processes in the medial basal hypothalamus, particularly regarding the control of PRL secretion. In addition, the cannabinoid-induced inhibition of LH secretion has been proposed to be mediated through changes in the hypothalamic mechanisms, namely opioid, noradrenergic, and dopaminergic influences, that control the release of gonadotrophin-releasing hormone to the portal blood [2, 22]. For instance, we have recently found that, in parallel to decreased LH levels, Δ^9-THC decreases hypothalamic noradrenergic activity while increasing dopaminergic tone [19], whereas Murphy *et al.* [22] have demonstrated that Δ^9-THC interferes with the noradrenergic response involved in the LH surge that occurs during sexual motivation.

To our knowledge, no data concerning the potential involvement of hypothalamic GABA-containing neurons in the effects of cannabinoids exist, although a tuberoinfundibular GABAergic system (composed of both neurons that store both GABA and DA and neurons that only store GABA [23]) that would release GABA into the portal blood to inhibit the anterior pituitary gland from secreting PRL has previously been reported (for review, see [24]). This pathway represents the major, although not the only, source of hypothalamic GABAergic innervation [25]; tuberoinfundibular dopaminergic neurons are subjected, for instance, to a direct, inhibitory influence of GABA that is possibly of an extrahypothalamic origin [26] that, in turn, would increase PRL secretion [27]. In addition, GABA inputs, acting locally into the hypothalamus, have also been involved in the control of gonadotrophin release [28].

The present study was designed to examine whether changes in the inputs of GABA and/or DA in the medial basal hypothalamus might occur concomitantly with the effects on the secretion of PRL and gonadotrophin that result from the activation of CB_1 receptors. We also addressed a second objective: testing whether the prolonged activation of these receptors might induce tolerance, with regard to the decrease in PRL and LH release, and whether this potential tolerance might be related to an agonist-induced decrease in CB_1-receptor binding and/or mRNA expression. The rationale for this second experiment was based on previous evidence that other effects of cannabinoids, such as motor inhibition, hypothermia, analgesia, and others, wane significantly in magnitude after the chronic administration of cannabinoids and that this tolerance was originated by a down-regulation of CB_1 receptors in specific brain nuclei [29–31].

MATERIALS AND METHODS

Animals, Treatments, and Sampling

Male Wistar rats were housed from birth in a room with a controlled photoperiod (lights on: 8 a.m.–8 p.m.) and temperature (23 ± 1°). They had free access to standard food (Panlab) and water. As adults (>8 weeks; 250 ± 25 g), the animals were used for two different experiments (all experiments were conducted according to European Animal Care Guides).

In Experiment I, the animals were subjected to a single i.p. injection of SR141716 (3 mg/kg of weight) a specific CB_1-receptor antagonist that was kindly supplied by Sanofi Recherche [32] or vehicle (distilled water with one drop of Tween 80). Thirty minutes later, both SR141716- and vehicle-injected animals were submitted to a new treatment: injections of Δ^9-THC (5 mg/kg of weight), kindly supplied by the National Institute on Drug Abuse, or vehicle (Tween 80-saline solution). Animals were sacrificed at 60 min after the final injection. This time was chosen, according to our previously published report [8], as the most appropriate for blocking CB-receptors. After sacrificed, the trunk blood was collected in tubes containing 0.4 mL of 6% EDTA, immediately centrifuged, and the plasma removed and stored frozen at −40° until analysis of the PRL and gonadotrophin levels. The anterior pituitary gland was removed and stored frozen at −40° until the analysis of the GABA and DA contents. The brain was also removed, and the medial basal hypothalamus immediately dissected and stored frozen at −40° until the analysis of the GABA and DA contents. To examine the specificity of potential GABA changes, other brain areas, such as the limbic forebrain, striatum, and ventral midbrain, were also dissected and used for the analysis of GABA contents. In this report, only data on the GABA contents in the limbic forebrain will be presented because data on the striatum and ventral midbrain have already been published [33].

In experiment II, the animals were subjected to a single i.p. injection of AM356 (10 mg/kg of weight), synthesized as previously described [34], or vehicle (Tween 80-saline solution). This AM356 compound is a more stable analog of anandamide, and we have recently demonstrated that it produces an early, marked, and persistent inhibitory effect

on PRL and LH secretion [8], one that is greater than the biphasic effect of Δ^9-THC and the transient effect of anandamide [8]. It is thus the most suitable compound for the purpose of this experiment.

Animals were sacrificed 20 min after treatment (acute situation). An additional group of animals were subjected to daily injections of AM356 (10 mg/kg of weight) or vehicle (Tween 80-saline solution) for 5 days and sacrificed 20 min after the last injection (chronic situation). In both situations, after sacrifice the trunk blood was collected in tubes containing 0.4 mL of 6% EDTA, immediately centrifuged, and the plasma removed and stored frozen at −40° until analyzed for the PRL and gonadotrophin levels. Their brains were also quickly and carefully removed and rapidly frozen by immersion in a 2-methyl-butane dry ice bath. Samples were stored at −70° until processed for autoradiographic analysis.

Autoradiographic Techniques for CB_1-Receptor Binding and mRNA Levels

TISSUE PREPARATION. Coronal sections 20 μm thick were cut in a cryostat, according to the Paxinos and Watson atlas [35]. For this experiment, sections corresponding to plates 24 and 28 of the Paxinos and Watson atlas were chosen because they contain an important diversity of hypothalamic structures. Sections were thaw-mounted onto RNAse-free gelatin/chrome alum coated slides, dried briefly at 30°, and stored at −80° until used. For the identification of the different hypothalamic nuclei, sections adjacent to those used for autoradiographic analysis were stained with cresyl-violet and analyzed according to the Paxinos and Watson atlas [35].

AUTORADIOGRAPHY FOR CB_1-RECEPTOR BINDING. The protocol used was basically that described by Jansen *et al.* [36], with slight modifications [8, 31], using 1 nM [^{3}H]-WIN 55,212-2 (DuPont/NEN), in the absence or presence of 10 μM nonlabeled WIN 55,212-2 (RBI), to determine the total and nonspecific binding, respectively.

IN SITU HYBRIDIZATION FOR CB_1 RECEPTOR mRNA LEVELS. *In situ* hybridization was carried out according to the procedure previously described by Rubino *et al.* [37], with slight modifications [38]. For the hybridization, we used 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 (DuPont), 3′-end labeled with [^{35}S]-dATP (Amersham Ibérica).

HPLC Determinations

ANALYSIS OF GABA CONCENTRATIONS. Analyses of GABA contents in the dissected medial basal hypothalami, anterior pituitary glands, and limbic forebrains were carried out by HPLC with electrochemical detection according to the procedure described by Smith and Sharp [39], with slight modifications [33].

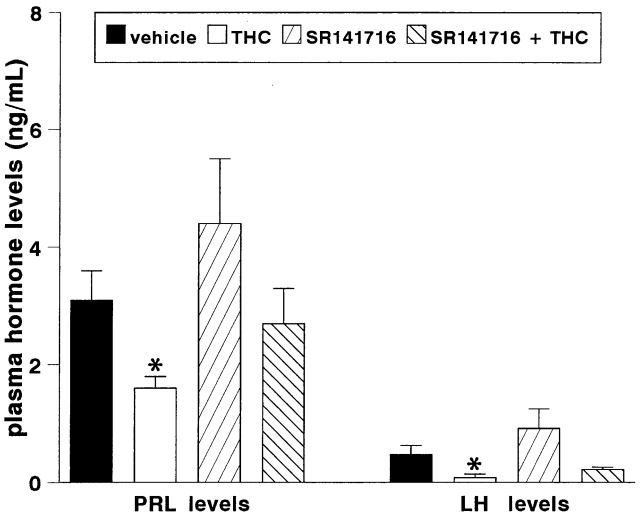

FIG. 1. Plasma PRL and LH levels of male rats subjected to an i.p. injection of Δ^9-THC, SR141716, or both and sacrificed at 60 min after the last injection (see details in the text). Values are means ± SEM of six determinations per group. Data were assessed by one-way ANOVA followed by the Student-Newman–Keuls test (*$P < 0.05$).

ANALYSIS OF DA CONCENTRATIONS. DA contents were analyzed using HPLC with electrochemical detection according to our previously published method [20].

Plasma PRL, LH and FSH Determinations

Plasma PRL, LH, and FSH levels were measured by specific double antibody radioimmunoanalysis systems using materials kindly supplied by the National Hormone and Pituitary Program. Details of these methods have previously been published [40, 41]. To avoid possible interassay variations, all samples were assayed in a single radioimmunoanalysis for each pituitary hormone. Plasma PRL, LH, and FSH levels were expressed as ng/mL of rPRL-RP3, rLH-RP3, and rFSH-RP2, respectively. The limits of detection were 0.025 pg/mL, 0.02 ng/mL and 0.3 ng/mL, respectively.

Statistics

Data from Experiment I (hormone levels, GABA and DA contents) were analyzed using a one-way ANOVA followed by the Student-Newman–Keuls test. Part of the data from Experiment II (receptor binding and hormone levels) were analyzed using a two-way ANOVA (treatment × duration), also followed by the Student-Newman–Keuls test, whereas data on mRNA levels were analyzed by the Student's *t*-test.

TABLE 1. Plasma FSH levels, DA contents in the medial basal hypothalamus and anterior pituitary gland, and GABA contents in the limbic forebrain

Groups	FSH levels (ng/mL)	DA contents (ng/mg tissue)		GABA contents (ng/mg tissue)
		Medial basal hypothalamus	Anterior pituitary gland	Limbic forebrain area
+ vehicle	10.72 ± 1.21	0.935 ± 0.159	0.240 ± 0.043	358.13 ± 23.46
+ Δ^9−THC	9.31 ± 1.37	1.043 ± 0.087	0.270 ± 0.084	357.40 ± 29.40
+ SR141716	8.36 ± 1.45	1.559 ± 0.322	0.225 ± 0.092	350.80 ± 27.29
+ SR141716 + Δ^9−THC	9.13 ± 1.50	1.027 ± 0.162	0.071 ± 0.048	375.27 ± 25.79

Male rats were subjected to an i.p. injection of (-)-Δ^9-THC or SR141716 or both and sacrificed at 60 min after the last injection (see details in the text). Values are means ± SEM of six determinations per group. Data were assessed by one-way ANOVA followed by the Student-Newman-Keuls test.

RESULTS

Experiment I: Involvement of Changes in GABA and/or DA Hypothalamic Inputs in Cannabinoid-induced Decreases in PRL and LH Secretion

As expected, the acute administration of Δ^9-THC produced a marked decrease in plasma PRL [$F(3, 20) = 2.997$, $P < 0.05$] and LH [$F(3, 17) = 3.055$, $P < 0.05$] levels (Fig. 1), with no changes in FSH [$F(3, 20) = 0.502$, not significant] levels (Table 1). This was paralleled by an increase in the contents of GABA (Fig. 2), but not of DA (Table 1), in the medial basal hypothalamus [GABA: $F(3, 18) = 4.993$, $P < 0.05$; DA: $F(3, 20) = 1.96$, not significant], whereas in the anterior pituitary gland only a nonsignificant increase in GABA could be observed [$F(3, 16) = 1.245$, not significant; Fig. 2], with no changes in DA [$F(3, 20) = 1.599$, not significant; Table 1]. The co-administration of Δ^9-THC and SR141716, a specific antagonist for CB_1 receptors, attenuated both the PRL and LH decreases (Fig. 1) and the GABA increase (Fig. 2) and produced a marked, but not statistically significant, reduction in the DA contents in the anterior pituitary gland (Table 1). SR141716 had no effect when administered alone (Fig. 2, Table 1). The specificity of the effects of Δ^9-THC on the GABA contents for the hypothalamic-anterior pituitary area can be confirmed because following the administration of this cannabinoid no changes in the GABA contents were seen in the limbic forebrain area [$F(3, 20) = 0.154$, not significant; see Table 1], as occurred in the striatum and the ventral midbrain [33].

Experiment II: Effects of Acute versus Chronic Exposure to AM356 on PRL and Gonadotrophin Levels and Hypothalamic CB_1-Receptor Binding and mRNA Expression

The chronic administration of AM356 produced a marked decrease in plasma PRL [treatment: $F(1, 28) = 9.54$, $P < 0.005$] and LH [treatment: $F(1, 30) = 10.87$, $P < 0.005$] levels, with no changes in FSH [treatment: $F(1, 29) = 0.09$, not significant; duration: $F(1, 29) = 0.59$, not significant; treatment × duration: $F(1,29) = 0.41$, not significant] (Fig. 3). The decrease in PRL levels was almost of similar

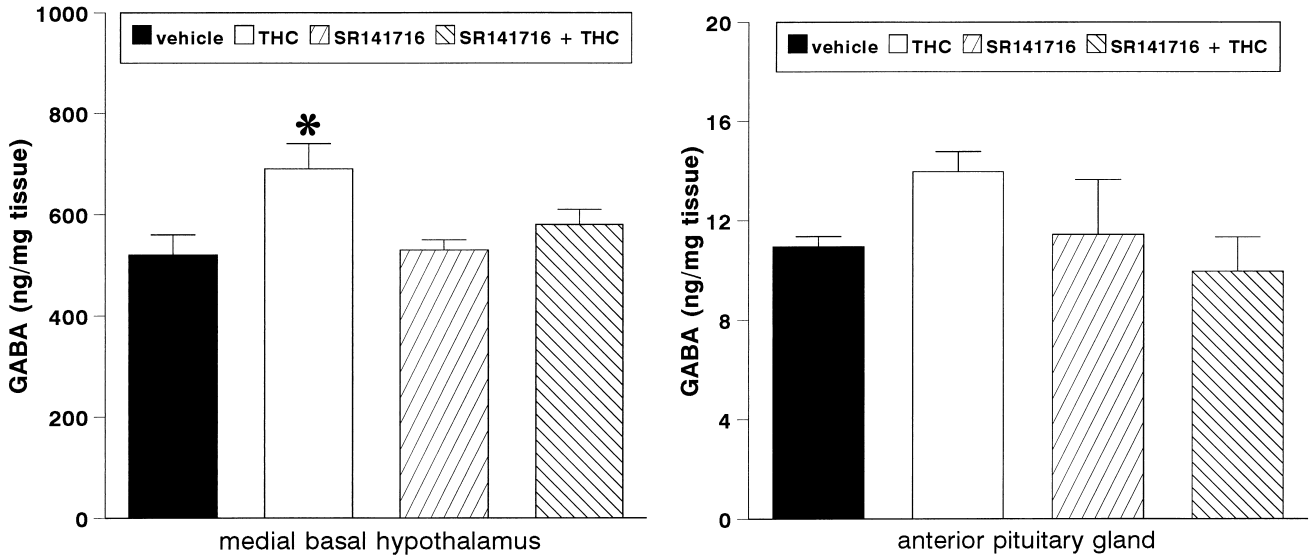

FIG. 2. GABA contents in the medial basal hypothalamus (left panel) and in the anterior pituitary gland (right panel) of male rats subjected to an i.p. injection of Δ^9-THC, SR141716, or both and sacrificed at 60 min after the last injection (see details in the text). Values are means ± SEM of six determinations per group. Data were assessed by one-way ANOVA followed by the Student-Newman–Keuls test (*$P < 0.05$).

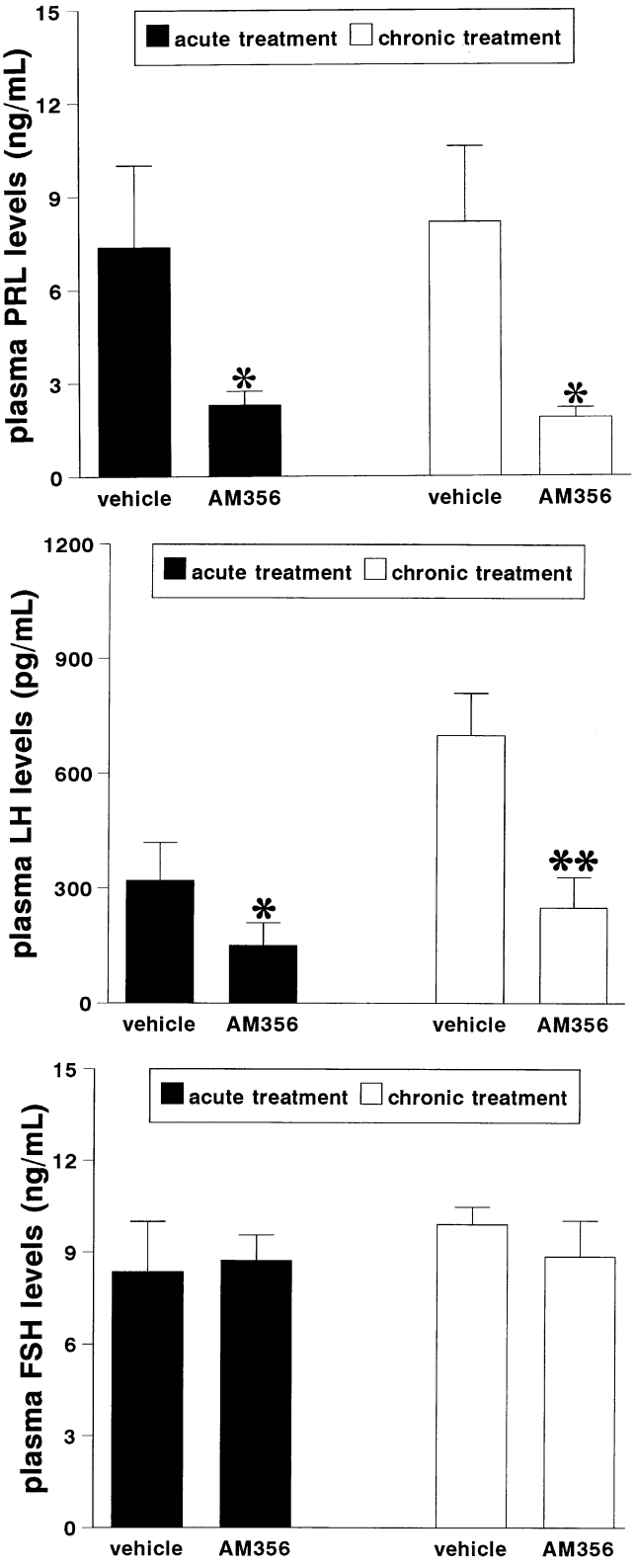

FIG. 3. Plasma PRL (top panel), LH (middle panel), and FSH (bottom panel) levels in male rats subjected to an acute or chronic (5 days) i.p. administration of AM356 or vehicle (Tween 80-saline). Animals were sacrificed at 20 min after the last injection (see details in the text). Values are means ± SEM of six determinations per group. Data were assessed by two-way ANOVA (treatment × duration) followed by the Student-Newman–Keuls test (*$P < 0.05$; **$P < 0.005$).

magnitude (−76.99%) to that caused by a single injection of this cannabimimetic compound (−68.93%) [duration: $F(1, 28) = 0.01$, not significant; treatment × duration: $F(1, 28) = 0.11$, not significant] (Fig. 3), thus suggesting the absence of tolerance. However, the decrease in LH levels was somewhat more pronounced in the chronic situation (−64.29%) than in the acute state (−53.13%), as was revealed by the trend observed in the two-way interaction [duration: $F(1, 30) = 6.72$, $P < 0.05$; treatment × duration: $F(1, 30) = 2.17$, $P = 0.151$]. This appears to be related to the higher LH levels measured in the vehicle-injected animals in the chronic situation than those in the vehicle-injected rats in the acute situation (Fig. 3).

Neither the acute nor the chronic administration of AM356 affected receptor binding in the ventromedial hypothalamic nucleus [treatment: $F(1, 12) = 0.37$, not significant; duration: $F(1, 12) = 0.53$, not significant; treatment × duration: $F(1, 12) = 0.28$, not significant] or the arcuate nucleus [treatment: $F(1, 12) = 2.50$, not significant; duration: $F(1, 12) = 0.48$, not significant; treatment × duration: $F(1, 12) = 0.21$, not significant] (Table 2). Only a slight decrease in receptor density could be observed in the arcuate nucleus of animals chronically exposed to AM356 (Table 2). mRNA levels for CB_1 receptor did not change in the ventromedial hypothalamic nucleus after the chronic AM356 exposure (Table 2). In the medial preoptic area, cannabinoid receptor binding did not change after treatment [treatment: $F(1, 12) = 0.67$, not significant; treatment × duration: $F(1, 12) = 0.41$, not significant]. However, there was statistical significance for the variable duration [$F(1, 12) = 8.10$, $P < 0.05$], thus reflecting higher levels of binding in animals acutely injected with AM356 or vehicle as compared with those chronically exposed (Table 2). This might be related to the fact that LH levels in animals that were chronically exposed to the vehicle were higher than in those acutely exposed (Fig. 3).

DISCUSSION

The present study confirms previous observations from our laboratory [3, 5–8, 17, 19–21] and others [1, 2, 4, 9, 10, 12, 18, 42, 43], which demonstrated that the administration of Δ^9-THC and related cannabinoids inhibits the secretion of PRL and LH. The present study provides new evidence that indicates that the effect of Δ^9-THC on PRL and LH release is caused by the activation of CB_1 receptors, which are expected to produce, among other effects, an enhancement of GABAergic activity in the hypothalamus-anterior pituitary axis. And this, in turn, might be related to the inhibition in PRL and LH secretion. Thus, the administration of a well-characterized dose of Δ^9-THC was followed 60 min later by a marked reduction in PRL and LH levels, with no changes in FSH. This was paralleled by an increase in the GABA contents in the medial basal hypothalamus and, to a lesser extent, in the anterior pituitary gland, indicating increased tuberoinfundibular GABAergic activ-

TABLE 2. Specific binding (fmol/mg tissue) and mRNA levels (arbitrary units of optical density) for cannabinoid receptors in several hypothalamic structures of adult male rats

Hypothalamic structures	Duration	Parameter	+vehicle	+AM356
Ventromedial hypothalamic nucleus	Acute	Specific binding (fmol/mg tissue)	22.98 ± 0.90	19.97 ± 1.41
	Chronic	Specific binding (fmol/mg tissue)	19.67 ± 4.38	19.44 ± 2.79
Arcuate nucleus	Acute	Specific binding (fmol/mg tissue)	17.79 ± 0.74	14.76 ± 2.29
	Chronic	Specific binding (fmol/mg tissue)	17.17 ± 3.02	11.63 ± 2.91
Medial preoptic area	Acute	Specific binding (fmol/mg tissue)	19.70 ± 0.74	19.97 ± 1.02
	Chronic	Specific binding (fmol/mg tissue)	14.35 ± 1.69	16.58 ± 1.81
Ventromedial hypothalamic nucleus	Chronic	mRNA levels (arbitrary units)	0.175 ± 0.024	0.174 ± 0.011

These were measured by autoradiography (specific binding) and *in situ* hybridization (mRNA levels) in rats acutely or chronically (5 days) exposed to AM356 or vehicle. Details in the text. Values are means ± SEM of six animals analyzed. Data were assessed by two-way ANOVA (treatment x duration) followed by the Student-Newman-Keuls test.

ity [25]. The GABA contents measured in both the medial basal hypothalamus and the anterior pituitary gland are similar to those reported previously by other authors [25, 44]. Because blockade of these receptors with SR141716 significantly waned both hormone decreases and GABA increases, it seems likely that this effect was originated through the activation of CB_1 receptors. In an earlier report, also using SR141716, we had already demonstrated the involvement of CB_1 receptors in the cannabinoid-induced inhibition of PRL release. This was observed again in the present study and extended to cannabinoid-induced inhibition in LH release and activation of GABAergic hypothalamic influences.

From the present data, it appears clear that, in absence of experiments with GABA receptor antagonists, we cannot state that the activation of GABAergic hypothalamic neurons mediates in the effects of cannabinoids on PRL and LH secretion. The only thing we can assert is that an increase in GABA transmission in the medial basal hypothalamus occurs in parallel with a decrease, caused by cannabinoids, in the secretion of these two hormones However, the possibility that CB_1 receptors could be located on GABA-containing neurons in the medial basal hypothalamus seems a priori attractive. In support of this possibility, it can be argued that hypothalamic deafferentation was not followed by changes in CB_1-receptor binding [45], thus suggesting that these receptors could be located in neurons intrinsic to the hypothalamus. Tuberoinfundibular GABAergic neurons are intrinsic to the hypothalamus [23, 25] and, hence, might be potential candidates. Along the same lines, Puder *et al.* [43] have observed that Δ^9-THC was also effective in acutely suppressing LH and PRL release in deafferentated animals, also supporting the notion that CB1 receptors could be located in neurons intrinsic to the hypothalamus. This is congruent with observations by Mailleux and Vanderhaeghen [46], who found that cell bodies of those neurons containing CB_1 receptors in the hypothalamus are located mainly in the ventromedial hypothalamic nucleus, as revealed by the location of their mRNA transcripts by in situ hybridization. However, CB_1-receptor mRNA expression was not detected in the arcuate nucleus [46], where cell bodies of tuberoinfundibular GABAergic neurons are located [23–25]. There also exist hypothalamic GABA-containing neurons other than tuberoinfundibular GABAergic neurons. However, these are extrinsic to the hypothalamus [26, 27] and are, therefore, unlikely to contain cannabinoid receptors.

Thus, the possibility that tuberoinfundibular GABAergic neurons might contain CB_1 receptors seems uncertain and will require further research. However, according to the present results, it is reasonable to conclude that the activation of these neurons appears to occur concomitantly to the effects of Δ^9-THC on PRL and LH secretion. Thus, activation of CB1 receptors by Δ^9-THC might produce, directly (if the receptors are located in GABA-containing neurons) or indirectly (through the activation of yet unknown neurons presumably connected with tuberoinfundibular GABAergic neurons), an increase in the synthesis and release of GABA from these neurons into the portal blood. This, in turn, would produce an increase in the contents of GABA in the anterior pituitary gland and inhibition of PRL secretion. Inhibition of LH secretion might also be produced through a direct effect of GABA on the gonadotroph cell. However, it appears more likely that this inhibition is originated through a GABA action that suppresses the activity of gonadotrophin-releasing hormone-containing neurons at the median eminence level [28].

The second objective of this study was to assess the possible existence of tolerance in the inhibitory effects of cannabinoids on the secretion of PRL and LH. Using a paradigm of five days of prolonged exposure with AM356, we did not observe attenuation in these inhibitory effects and, subsequently, did not find any alterations in CB1 receptor binding and mRNA expression in selected hypothalamic nuclei. Some changes were indeed observed, but they do not appear to be related to a pharmacological tolerance/receptor desensitization phenomenon. On the other hand, it cannot be argued that the absence of changes in CB_1 receptors might be related to a general inability of AM356 to alter CB_1-receptor binding and/or mRNA expression because, after 5 days of daily exposure, this compound produced significant decreases in these two parameters in other brain regions, such as the basal ganglia (caudate-putamen area, entopeduncular nucleus and substantia nigra), cerebellum, and hippocampal structures

(data not shown). In the present study, we paid special attention to the entopeduncular nucleus because it can be visualized in the same sections as most of the hypothalamic nuclei. In this nucleus, chronic AM356 exposure produced a statistically significant decrease in CB_1- receptor binding (approx. −22.1%).

In summary, the activation of CB_1 receptors in hypothalamic nuclei produced the expected decreases in PRL and LH secretion, which occurred concomitantly with an increase in GABAergic activity in the hypothalamus-anterior pituitary axis. The prolonged activation of these receptors for 5 days did not elicit tolerance in terms of an attenuation in the magnitude of the decrease in PRL and LH and did not alter the levels of CB_1-receptor binding and mRNA in several hypothalamic nuclei.

This work was supported by grants from Comision Interministerial de Ciencia y Tecnologia (PM96-0049: R. M., J. R., L. G. G., S. G., J. A. R. and J. J. F. R.), Fondo de Investigaciones Sanitarias (Spain) (94/0299: R. M. M. and M. A. V.) and National Institute on Drug Abuse (NIDA) (DA 3801 and DA 9158: A. M.). The authors are indebted to Sanofi Recherche for the gift of SR141716, the NIDA for kindly supplying Δ^9-THC, and to the National Institutes of Health for providing the reagents for PRL, LH and FSH assays.

References

1. Murphy LL, Steger RW, Smith MS and Bartke A, Effects of Δ^9-tetrahydrocannabinol, cannabinol and cannabidiol, alone and in combination, on luteinizing hormone and prolactin release and on hypothalamic neurotransmitters in the male rat. *Neuroendocrinology* **52**: 316–321, 1990.
2. Murphy LL, Steger RW and Bartke A, Psychoactive and non-psychoactive cannabinoids and their effects on reproductive neuroendocrine parameters. In: *Biochemistry and Physiology of Substance Abuse*, vol. 2. Ed. Watson RR, pp. 73–93. CRC Press, Boca Raton, FL, 1990.
3. Rodriguez de Fonseca F, Fernandez-Ruiz JJ, Murphy LL, Cebeira M, Steger RW, Bartke A and Ramos JA, Acute effects of Δ^9-tetrahydrocannabinol on dopaminergic activity in several rat brain areas. *Pharmacol Biochem Behav* **42**: 269–275, 1992.
4. Dalterio SL, Cannabinoid exposure: Effects on development. *Neurobehav Toxicol Teratol* **8**: 345–352, 1986.
5. Rodriguez de Fonseca F, Cebeira M, Fernandez-Ruiz JJ, Navarro M and Ramos JA, Effects of pre- and perinatal exposure to hashish extracts on the ontogeny of brain dopaminergic neurons. *Neuroscience* **43**: 713–723, 1991.
6. Fernandez-Ruiz JJ, Rodriguez de Fonseca F, Navarro M and Ramos JA, Maternal cannabinoid exposure and brain development: Changes in the ontogeny of dopaminergic neurons. In: *Marihuana/Cannabinoids: Neurobiology and Neurophysiology*, Eds. Murphy LL and Bartke A, pp. 119–164. CRC Press, Boca Raton, FL, 1992.
7. Romero J, GarcRa-Gil L, Ramos JA and Fernandez-Ruiz JJ, The putative cannabinoid receptor ligand, anandamide, stimulates tyrosine hydroxylase activity and inhibits prolactin release. *Neuroendocrine Lett* **16**: 159–164, 1994.
8. Fernandez-Ruiz JJ, Munoz RM, Romero J, Villanua MA, Makriyannis A and Ramos JA, Time-course of the effects of different cannabimimetics on prolactin and gonadotrophin secretion: Evidence for the presence of CB_1 receptors in hypothalamic structures and their involvement in the effects of cannabimimetics. *Biochem Pharmacol* **53**: 1919–1927, 1997.
9. Wenger T, Fragakis G, Probonas K, Toth BE and Yiannakakis N, Anandamide (endogenous cannabinoid) affects anterior pituitary hormone secretion in adult male rats. *Neuroendocrine Lett* **16**:295–303, 1994.
10. Wenger T, Jamali KA, Juanede C, Leonardelli J and Tramu G, Arachidonyl ethanolamide (anandamide) activates the parvocellular part of hypothalamic paraventricular nucleus. *Biochem Biophys Res Comm* **237**: 724–728, 1997.
11. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R, Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**: 1946–1949, 1992.
12. Hughes CL Jr, Everett JW. and Tyrey L, Δ^9-tetrahydrocannabinol suppression of prolactin secretion in the rat: Lack of direct pituitary effect. *Endocrinology* **109**: 876–880, 1981.
13. Murphy LL, Newton SC, Dhali J and Chavez D, Evidence for a direct anterior pituitary site of Δ^9-tetrahydrocannabinol action. *Pharmacol Biochem Behav* **40**: 603–608, 1991.
14. Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR and Rice KC, Characterization and localization of cannabinoid receptors in rat brain: A quantitative *in vitro* autoradiographic study. *J Neurosci* **11**: 563–583, 1991.
15. Howlett AC, Bidaut-Russell M, Devane WA, Melvin LS, Johnson MR and Herkenham M, The cannabinoid receptor: biochemical, anatomical and behavioral characterization. *Trends Neurosci* **13**: 420–423, 1990.
16. Lynn AB and Herkenham M, Localization of cannabinoid receptors and nonsaturable high-density cannabinoid binding sites in peripheral tissues of the rat: Implications for receptor-mediated immune modulation by cannabinoids. *J Pharmacol Exp Ther* **268**: 1612–1623, 1994.
17. Bonnin A, Ramos JA, Rodriguez de Fonseca F, Cebeira M and Fernandez-Ruiz JJ, Acute effects of Δ^9-tetrahydrocannabinol on tuberoinfundibular dopaminergic activity, anterior pituitary sensitivity to dopamine and prolactin release vary as a function of estrous cycle. *Neuroendocrinology* **58**: 280–286, 1993.
18. Kramer J and Ben-David M, Prolactin supression by (-)-Δ^9-tetrahydrocannabinol (THC): Involvement of serotoninergic and dopaminergic pathways. *Endocrinology* **103**: 452–458, 1978.
19. Fernandez-Ruiz JJ, Navarro M, Hernandez ML, Vaticn D and Ramos JA, Neuroendocrine effects of an acute dose of Δ^9-tetrahydrocannabinol: changes in hypothalamic biogenic amines and anterior pituitary hormone secretion. *Neuroendocrine Lett* **14**: 349–355, 1992.
20. Bonnin A, de Miguel R, Fernandez-Ruiz JJ, Cebeira M and Ramos JA, Possible role of the cytochrome P450-linked monooxygenase system in preventing Δ^9-tetrahydrocannabinol-induced stimulation of tuberoinfundibular dopaminergic activity in female rats. *Biochem Pharmacol* **48**: 1387–1392, 1994.
21. Bonnin A, Fernandez-Ruiz JJ, Cebeira M and Ramos JA, Effects of Δ^9-tetrahydrocannabinol on tuberoinfundibular dopaminergic activity and prolactin release in estrogen-replaced ovariectomized rats. *Neuroendocrine Lett* **16**: 305–314, 1994.
22. Murphy LL, Chandrashekar V and Bartke A, Δ^9-Tetrahydrocannabinol inhibits luteinizing hormone secretion in the male rat: effect of intracerebroventricular norepinephrine infusion. *Neuroendocrine Lett* **16**: 1–7, 1994.
23. Schimchowitsch S, Vuillez P, Tappaz ML, Klein MJ and Stoeckel ME, Systemic presence of GABA-immunoreactivity in the tubero-infundibular and tubero-hypophyseal dopaminergic axonal systems: An ultrastructural immunogold study on several mammals. *Exp Brain Res* **83**: 575–586, 1991.
24. Racagni G, Apud JA, Cocchi D, Locatelli V and Muller EE,

GABAergic control of anterior pituitary hormone secretion. *Life Sci* **31**: 823–838, 1982.
25. Nicoletti F, Grandison L and Meek JL, Effects of repeated administration of estradiol benzoate on tubero-infundibular GABAergic activity in male rats. *J Neurochem* **44**: 1217–1220, 1985.
26. Sugita S, Johnson SW and North RA, Synaptic inputs to $GABA_A$ and $GABA_B$ receptors originate from discrete afferent neurons. *Neurosci Lett* **134**: 207–211, 1992.
27. Wagner EJ, Goudreau JL, Moore KE and Lookingland KJ, GABAergic regulation of tuberoinfundibular dopaminergic neurons in the male rat. *Brain Res* **659**: 194–200, 1994.
28. Tal J, Price MT and Olney JW, Neuroactive amino acids influence gonadotrophin output by a suprapituitary mechanism in rodents or primates. *Brain Res* **273**: 179–182, 1983.
29. Oviedo A, Glowa J and Herkenham M, Chronic cannabinoid administration alters cannabinoid receptor binding in rat brain: A quantitative autoradiographic study. *Brain Res* **616**: 293–302, 1993.
30. Sim LJ, Hampson RE, Deadwyler SA and Childers SR, Effects of chronic treatment with Δ^9-tetrahydrocannabinol on cannabinoid-stimulated [^{35}S]γ autoradiography in rat brain. *J Neurosci* **15**: 8057–8066, 1996.
31. Romero J, Garcia-Palomero E, Castro JG, Garcia-Gil L, Ramos JA and Fernandez-Ruiz JJ, Effects of chronic exposure to Δ^9-tetrahydrocannabinol on cannabinoid receptor binding and mRNA levels in several rat brain regions. *Brain Res Mol Brain Res* **46**: 100–108, 1997.
32. Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J, Neliat G, Caput D, Ferrara P, SoubriJ P, BreliPre JC and Le Fur G, SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350**: 240–244, 1994.
33. Romero J, de Miguel R, Ramos JA and Fernandez-Ruiz JJ, The activation of cannabinoid receptors in striatonigral GABAergic neurons inhibited GABA uptake. *Life Sci* **62**: 351–363, 1998.
34. Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG and Makriyannis A, (R)-Methanandamide: A chiral novel anandamide possessing higher potency and metabolic stability. *J Med Chem* **37**: 1889–1893, 1994.
35. Paxinos G and Watson C, *The rat brain in stereotaxic coordinates*. Academic Press, London, 1986.
36. Jansen EM, Haycock DA, Ward SJ and Seybold US, Distribution of cannabinoid receptors in rat brain determined with aminoalkylindoles. *Brain Res* **575**: 93–102, 1992.
37. Rubino T, Massi P, Patrini G, Venier I, Giagnoni G and Parolaro D, Chronic CP-55,940 alters cannabinoid receptor mRNA in the rat brain: An *in situ* hybridization study. *Neuroreport* **5**: 2493–2496, 1994.
38. Romero J, Berrendero F, Garcia-Gil L, de la Cruz P, Ramos JA and Fernandez-Ruiz JJ, Loss of cannabinoid receptor binding and messenger RNA levels and cannabinoid agonist-stimulated [^{35}S]guanylyl-5′0-(thio)-triphosphate binding in the basal ganglia of aged rats. *Neuroscience* **84**: 1075–1083, 1998.
39. Smith S and Sharp T, Measurement of GABA in rat brain microdialysates using o-phthaldialdehyde-sulphite derivatization and high-performance liquid chromatography with electrochemical detection. *J Chromatography* **652**: 228–233, 1994.
40. Esquifino AI, Marc I and Lafuente A, Cyclosporine modifies the pulsatile secretory patterns of prolactin and luteinizing hormone in normal and pituitary-grafted female rats. *Neuroendocrinology* **60**: 581–588, 1994.
41. Esquifino AI, Moreno ML, Agrasal C and Villanua MA, Effects of cyclosporine on ovarian function in sham-operated and pituitary-grafted young female rats. *Proc Soc Exp Biol Med* **208**: 397–403, 1995.
42. Tuomisto J and Mannisto P, Neurotransmitter regulation of anterior pituitary hormones. *Pharmacol Rev* **37**: 249–332, 1985.
43. Puder M, Nir I and Siegel RA, The effect of Δ^1-tetrahydrocannabinol on luteinizing hormone release in castrated and hypothalamic deafferented male rats. *Exp Brain Res* **59**: 213–216, 1985.
44. Müller EE, Some aspects of the neurotransmitter control of anterior pituitary function. *Pharmacol Res* **21**: 75–85, 1989.
45. Romero J, Wenger T, de Miguel R, Ramos JA and Fernandez-Ruiz JJ, Cannabinoid receptor binding did not vary in several hypothalamic nuclei after hypothalamic deafferentation. *Life Sci* **63:** 351–356, 1998.
46. Mailleux Pand Vanderhaeghen JJ, Distribution of neuronal cannabinoid receptors in the adult rat brain: A comparative receptor binding radioautography and *in situ* hybridization histochemistry. *Neuroscience* **48**: 655–668, 1992.