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Time-Course of the Cannabinoid Receptor Down-Regulation in the Adult Rat Brain Caused by Repeated Exposure to Δ^9-Tetrahydrocannabinol

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KEY WORDS cannabinoid receptor binding; cannabinoid receptor mRNA levels; Δ^9-tetrahydrocannabinol; autoradiography; in situ hybridization; chronic exposure; down-regulation

ABSTRACT Recent studies have demonstrated that the pharmacological tolerance observed after prolonged exposure to plant or synthetic cannabinoids in adult individuals seems to have a pharmacodynamic rather than pharmacokinetic basis, because down-regulation of cannabinoid receptors was assessed in the brain of cannabinoid-tolerant rats. In the present study, we have examined the time-course of cannabinoid receptor down-regulation by analyzing cannabinoid receptor binding, using autoradiography, and mRNA expression, using in situ hybridization, in several brain structures of male adult rats daily exposed to Δ^9-tetrahydrocannabinol (Δ^9-THC) for 1, 3, 7, or 14 days. With only the exception of a few number of areas, most of the brain regions exhibited a progressive decrease in cannabinoid receptor binding. Two facts deserve to be mentioned. First, the pattern of this down-regulation process presented significant regional differences in terms of onset of the decrease and magnitude reached. Second, the loss of cannabinoid receptor binding was usually accompanied by no changes in its mRNA expression. Thus, some structures, such as most of the subfields of the Ammon's horn and the dentate gyrus in the hippocampus, exhibited a rapid (it appeared after the first injection) and marked (it reached approximately 30% of decrease after 14 days) reduction of cannabinoid receptor binding as a consequence of the daily Δ^9-THC administration. However, no changes occurred in mRNA levels. Decreased binding was also found in most of the basal ganglia, but the onset of this reduction was slow in the lateral caudate-putamen and the substantia nigra (it needed at least three days of daily Δ^9-THC administration), and, in particular, in the globus pallidus (more than 3 days). The magnitude of the decrease in binding was also more moderate, with maximal reductions always less than 28%. No changes were seen in the entopeduncular nucleus and only a trend in the medial caudate-putamen. However, the decrease in binding in some basal ganglia was, in this case, accompanied by a decrease in mRNA levels in the lateral caudate-putamen, but this appeared after 7 days of daily Δ^9-THC administration and, hence, after the onset of binding decrease. In the limbic structures, cannabinoid receptor binding decreased in the septum nuclei (it needed at least 3 days of daily Δ^9-THC administration), tended to diminish in the nucleus accumbens and was unaltered in the basolateral amygdaloid nucleus, with no changes in mRNA levels in these last two regions. Binding also decreased in the superficial and deep layers of the cerebral cortex, but only accompanied

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by trends in mRNA expression. The decrease in binding was initiated promptly in the deep layer (after the first injection) and it reached more than 30% of reduction after 14 days of daily Δ^9-THC administration, whereas, in the superficial layer, it needed more than 3 days of daily Δ^9-THC administration and reached less than 30% of reduction. Finally, no changes in binding and mRNA levels were found in the ventromedial hypothalamic nucleus. In summary, the daily administration of Δ^9-THC resulted in a progressive decrease in cannabinoid receptor binding in most of the brain areas studied, and it was a fact that always occurred before the changes in mRNA expression in those areas where these existed. The onset of the decrease in binding exhibited regional differences with areas, such as most of the hippocampal structures and the deep layer of the cerebral cortex, where the decrease occurred after the first administration. Other structures, however, needed at least 3 days or more to initiate receptor binding decrease. Two structures, the entopeduncular nucleus and the ventromedial hypothalamic nucleus, were unresponsive to chronic Δ^9-THC administration, whereas others, the medial caudate-putamen and the basolateral amygdaloid nucleus, only exhibited trends. **Synapse 30:298–308, 1998.**

INTRODUCTION

Recent studies (Abood et al., 1993; Oviedo et al., 1993; Rodríguez de Fonseca et al., 1994; Romero et al., 1995, 1997; Rubino et al., 1994; Sim et al., 1996; Westlake et al., 1991) addressed the molecular events underlying the pharmacological tolerance observed for a variety of effects (motor inhibition, analgesia, hypothermia, hypotension) after a prolonged exposure to cannabinoids in adult individuals (for review, see Dewey, 1986; Pertwee, 1991, 1995). Thus, despite some reports that showed no changes in cannabinoid receptor binding or mRNA expression (Abood et al., 1993; Westlake et al., 1991), recent studies from our group (Rodríguez de Fonseca et al., 1994; Romero et al., 1995, 1997) and others (Oviedo et al., 1993; Rubino et al., 1994; Sim et al., 1996) have clearly demonstrated that pharmacological tolerance appears to be linked more to changes in cannabinoid receptor availability, rather than to changes in cannabinoid metabolism. Oviedo et al. (1993), using autoradiographic techniques, and our group, using binding of [^{3}H]-CP55,940 to brain membranes (Rodríguez de Fonseca et al., 1994; Romero et al., 1995) and, more recently, autoradiography (Romero et al., 1997), have demonstrated that cannabinoid receptor binding in several brain structures, mainly in extrapyramidal areas, decreases after chronic cannabinoid exposure in rats. This fact runs in parallel to a pronounced reduction in the magnitude of motor inhibition caused by an acute cannabinoid treatment. Of particular interest is the report from Oviedo et al. (1993), who clearly proved down-regulation of cannabinoid receptors, by using regional Kd/Bmax analysis, after chronic cannabinoid exposure. In addition, Rubino et al. (1994) have also recently demonstrated that mRNA levels of cannabinoid CB_1 receptor subtype in the caudate-putamen area, measured by in situ hybridization, decreased, in parallel to motor tolerance, after chronic treatment with CP55,940, with no alterations in other brain areas. More recently, Sim et al. (1996) also reported the existence of a profound desensitization of cannabinoid-activated signal transduction mechanisms, using autoradiographic analysis of WIN-55,212–2-stimulated [^{35}S]-guanylyl-5'-*O*-(γ-thio)-triphosphate ([^{35}S]-GTPγS) binding, in Δ^9-tetrahydrocannabinol (Δ^9-THC)-tolerant rats. In this study, the regions particularly affected were the hippocampus and the basal ganglia.

Despite these recent studies that support a pharmacodynamic basis for cannabinoid tolerance, several aspects remain to be clarified. An important question is the elucidation of the onset of cannabinoid receptor down-regulation, which probably will present regional differences, and the parallelism between changes in binding and in mRNA expression. To our knowledge, the only study on this aspect has been recently presented by Breivogel et al. (1997) using WIN-55,212–2-stimulated [^{35}S]-GTPγS binding. These authors found that desensitization occurred slowly in some areas, such as the globus pallidus and the cerebellum, whereas in others, such as the hippocampus, the effect was more rapid. Our present study has been designed to further explore the time-course of cannabinoid receptor down-regulation in several brain structures. To this end, we have analyzed cannabinoid receptor binding, by using autoradiography, and mRNA expression, by using in situ hybridization, in slide-mounted brain sections obtained from adult male rats that had been daily exposed to Δ^9-THC for 1, 3, 7, or 14 days.

MATERIALS AND METHODS

Animals, treatments, and sampling

Male Wistar rats were housed from birth in a room with controlled photoperiod (08:00–20:00 hours light) and temperature (23 ± 1ºC). They had free access to standard food (Panlab, Barcelona, Spain) and water. Animals were used for experimental purposes at adult age (> 8 weeks of life). Animals were submitted to a daily i.p. administration of Δ^9-THC (5 mg/kg body weight/day), kindly supplied from the National Insti-

tute on Drug Abuse (Rockville, MD, USA), during 1, 3, 7, or 14 days. As controls, animals i.p. injected with vehicle (Tween-saline solution) for a period of 7 days were used. In all the cases, it is important to remark that animals were sacrificed at 24 hours after the last injection. After sacrifice, brains were quickly and carefully removed and rapidly frozen by immersion in 2-methyl-butane cold in dry ice. All samples were stored at -70ºC until processed. Coronal sections 20 µm-thick were cut in a cryostat, according to the Paxinos and Watson atlas (1986). Sections were thaw-mounted onto RNAse-free gelatin/chrome alum coated slides and dried briefly at 30ºC and stored at -80ºC until used. For the identification of the different brain nuclei, adjacent sections to those used for autoradiographic analysis were stained with cresyl-violet and analyzed according to the Paxinos and Watson atlas (1986). Sections from 8–9 different animals *per* group (days of treatment) were used for each of the two autoradiographic analyses done.

Autoradiography of cannabinoid receptor binding

The protocol used is basically the method described by Jansen et al. (1992) with modifications. Briefly, slide-mounted brain sections were preincubated for 20 minutes, at 30ºC, in a buffer containing 20 mM HEPES with 0.5% bovine serum albumin (fatty acid-free), pH 7.0. Slides were then incubated for 80 minutes, at 30ºC, with 1 nM [^{3}H]- WIN 55,212–2 (Du Pont NEN, ITISA, Madrid, Spain) prepared in the same buffer, in the absence or the presence of 10 µM non-labelled WIN 55,212–2 (RBI, Natick, MA) to determine the total and the non-specific binding, respectively. Following this incubation, slides were washed in buffer four times (10 minutes each) at room temperature, dipped in ice-cold distilled water, and then dried under a stream of cool dried air. Autoradiograms were generated by apposing the labelled tissues, together with autoradiographic standards ([^{3}H] micro-scales, Amersham Ibérica, Madrid, Spain), to tritium-sensitive film ([^{3}H]-Hyperfilm, Amersham Ibérica) for a period of 3–4 weeks, and developed (D-19, Kodak, Rochester, NY) for 4 minutes at 20ºC. Developed films were analyzed and quantitated in a computer-assisted videodensitometer (Image Quant 3.3, Molecular Dynamics, Krefeld, Germany) using the standard curve generated from [^{3}H]-standards. In this sense, after logarithmic transformation, all data were best fitted to a linear equation. The range of standards used assured linearity between the following interval: 20–500 fmol/mg tissue.

Analysis of cannabinoid receptor mRNA levels by in situ hybridization

In situ hybridization was carried out according to the procedure previously described by Rubino et al. (1994) with slight modifications. Briefly, sections were fixed in 4% formaldehyde for 5 minutes and, after rinsing twice in phosphate buffer saline, were acetylated by incubation in 0.25% acetic anhydride, prepared in 0.1M triethanolamine/0.15M sodium chloride (pH 8.0), for 10 minutes. Sections were rinsed in 0.3M sodium chloride/0.03M 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, data not shown) was 3'-end labelled with [^{35}S]-dATP (Amersham Ibérica,) using terminal deoxynucleotidyl-transferase (Boehringer Mannheim, Barcelona, Spain). Sections were, then, hybridized with [^{35}S]-labelled oligonucleotide probes (2.5×10^5 dpm *per* section), washed, and exposed to X-ray film (βmax, Amersham Ibérica) for 10 days, and developed (D- 19, Kodak) for 6 minutes at 20ºC. The intensity of the hybridization signal was assessed by measuring the grey levels in the autoradiographic films with a computer-assisted videodensitometer (Image Quant 3.3, Molecular Dynamics). Additional brain sections were co-hybridized with a 100-fold excess of cold probe or with RNAse to assert the specificity of the signal. As expected, no hybridization signal was detected in these sections (data not shown).

Statistics

Data on cannabinoid receptor binding and mRNA levels were assessed by one-way analysis of variance followed by Student-Newman-Keuls test.

RESULTS

Basal ganglia

Specific binding

The one-way analysis of variance of the data in the basal ganglia revealed that the daily administration of Δ^9-THC produced a marked decrease of cannabinoid receptor binding in the lateral caudate-putamen ($F(4,36) = 4.659$, $P < 0.005$), substantia nigra ($F(4,36) = 3.159$, $P < 0.05$), and globus pallidus ($F(4,36) = 3.397$, $P < 0.05$), whereas a certain trend to decrease was seen in the medial caudate-putamen ($F(4,36) = 2.032$, $P = 0.111$) and no changes in the entopeduncular nucleus ($F(4,36) = 0.765$, ns). The post-hoc analysis revealed that the decrease in cannabinoid receptor binding did not reach statistical significance up to 3 days of daily Δ^9-THC treatment in the lateral caudate-putamen (-11.6%) and substantia nigra (-23.5%) (Fig. 1). In the lateral caudate-putamen, the decrease was stressed after 7 and 14 days of daily Δ^9-THC treatment (-19.4% and -20.6%, respectively) (Fig. 1), but this did not occur in the substantia nigra, where the decrease in binding was higher after 7 days (-27.1%) than after 14 days (-17.4%) (Fig. 1). In the globus pallidus, the post-hoc

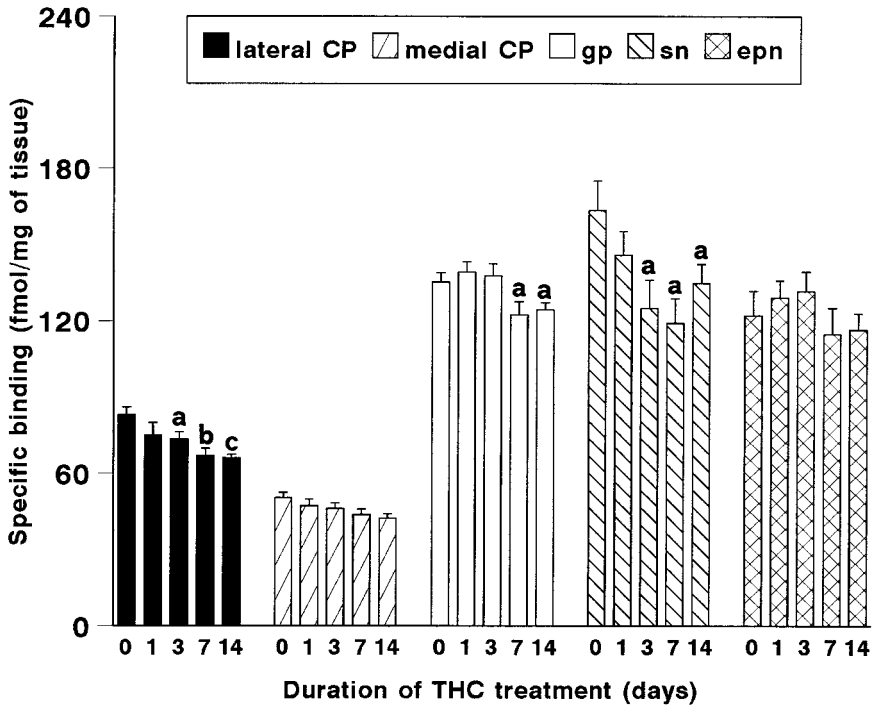

Fig. 1. Specific binding (fmol/mg tissue) for cannabinoid receptors measured by autoradiography in the lateral and medial caudate-putamen (CP), globus pallidus (gp), substantia nigra (sn), and entopeduncular nucleus (epn) of adult male rats daily exposed to Δ^9-tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls (0) were injected 7 days with vehicle. Details are in the text. Values are means ± SEM of 8–9 determinations *per* group. Data were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test (a: $P < 0.05$; b: $P < 0.005$; c: $P < 0.0005$).

analysis revealed that only after 7 and 14 days of daily Δ^9-THC treatment could a statistically significant decrease be observed, although very small in both cases (-9.5% and -8.0%, respectively) and presumably, unrelevant from a physiological point of view (Fig. 1). Finally, a trend to progressively decrease was observed in the medial caudate-putamen, although without reaching statistical significance, as mentioned above (Fig. 1). Representative autoradiograms used for the quantification of specific binding in the basal ganglia of Δ^9-THC-exposed and control rats can be seen in Figure 2.

mRNA levels

The decrease in binding observed in the lateral caudate-putamen was accompanied by a parallel decrease in mRNA levels, as the statistical significance revealed in this area when data were assessed by one-way analysis of variance ($F(4,32) = 4.525$, $P < 0.005$). The post-hoc analysis revealed that the decrease was only statistically significant after 7 and 14 days of daily Δ^9-THC administration (-40.5% and -31.7%, respectively) (Fig. 3). In the case of the medial caudate-putamen, only a trend to decrease was observed ($F(4,32) = 2.013$, $P = 0.116$), which coincides with that observed for binding in this area. This trend to decrease was observed only after 7 and 14 days of daily Δ^9-THC administration (-29.5% and -24.8%, respectively) (Fig. 3), which correlates with data in the lateral region of the caudate-putamen. Representative autoradiograms used for the quantification of mRNA levels in the caudate-putamen of Δ^9-THC-exposed and control rats can be seen in Figure 2.

Hippocampus

Specific binding

The daily administration of Δ^9-THC also produced a marked decrease of cannabinoid receptor binding in the dentate gyrus ($F(4,35) = 5.702$, $P < 0.005$) and the different subfields of the Ammon's horn (CA1: $F(4,35) = 12.865$, $P < 0.00005$; CA2: $F(4,35) = 7.893$, $P < 0.0005$; CA3: $F(4,35) = 9.047$, $P < 0.00005$; CA4: $F(4,35) = 4.343$, $P < 0.005$). The decrease in cannabinoid receptor binding already appeared 24 hours after the first Δ^9-THC injection in the CA1 (-12.3%), CA2 (-11.4%), and CA3 (-14.9%) subfields and in the dentate gyrus (-10.6%) (Fig. 4). It was progressively more marked after 3 days (CA1: -23.0%; CA2: -18.0%; CA3: -20.0%; dentate gyrus: -12.0%) and 7 days (CA1: -31.6%; CA2: -28.7%; CA3: -30.3%; dentate gyrus: -26.4%) of daily Δ^9-THC treatment, and reached a maximum after 14 days (CA1: -34.8%; CA2: -31.4%; CA3: -32.4%; dentate gyrus: -27.7%) (Fig. 4). The CA4 subfield of the Ammon's horn also reached a maximum after 7 and 14 days of daily Δ^9-THC treatment (-25.7 and -30.3%, respec-

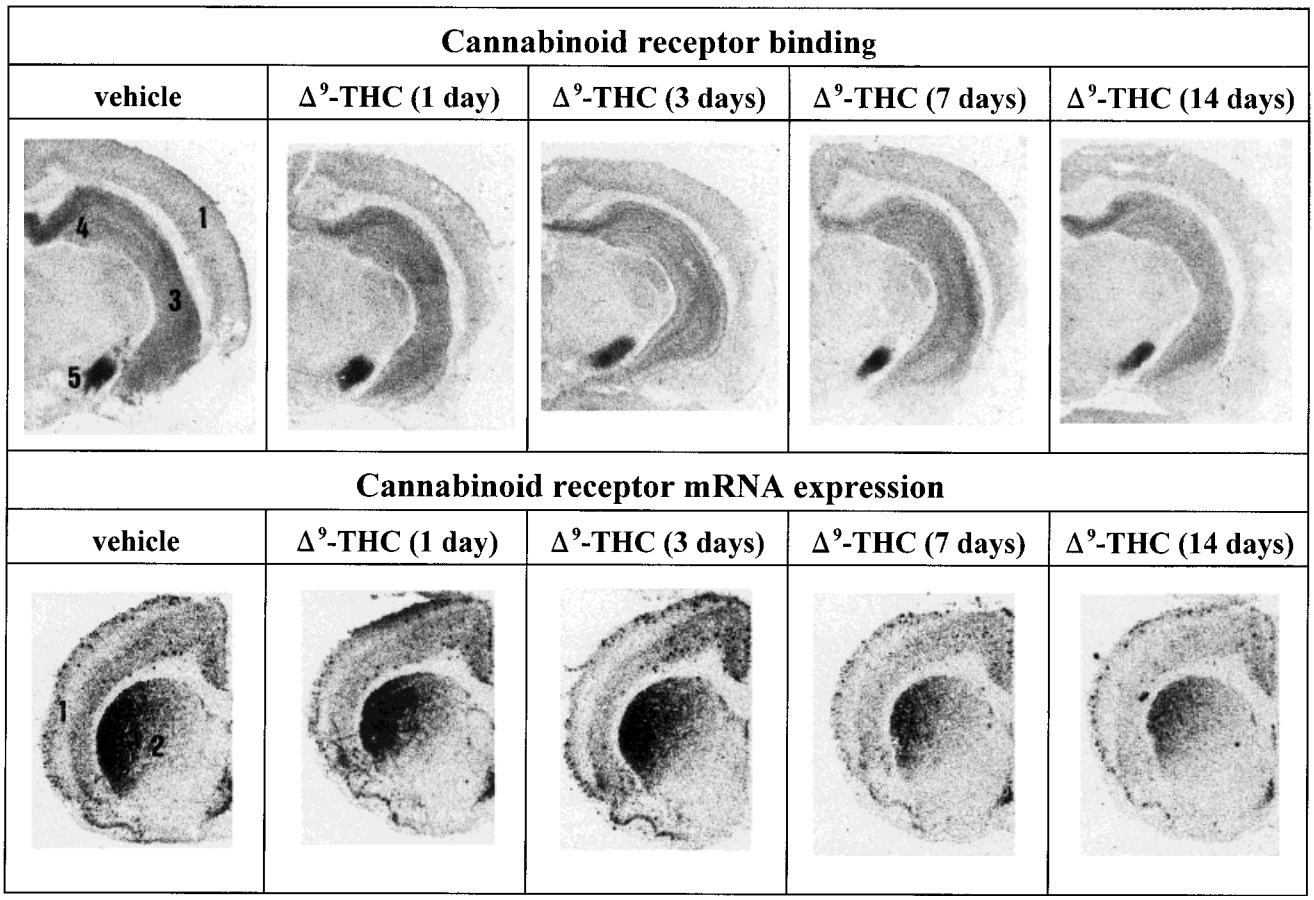

Fig. 2. Representative autoradiograms for cannabinoid receptor binding (top) and mRNA expression (bottom) corresponding to plates 37 and 16, respectively, according to the Paxinos and Watson atlas, obtained from slide-mounted sections of adult male rats chronically exposed to Δ^9- tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls were injected 7 days with vehicle. Autoradiograms were processed according to the conditions described in Materials and Methods. 1: cerebral cortex; 2: caudate-putamen; 3: Ammon's horn; 4: dentate gyrus; 5: substantia nigra.

tively), but the decreases after 1 and 3 days (-8.2% and -14.4%, respectively) were not statistically significant (Fig. 4). Representative autoradiograms used for the quantification of specific binding in the hippocampal structures of Δ^9-THC-exposed and control rats can be seen in Figure 2.

mRNA levels

The changes observed in cannabinoid receptor binding in the different structures of the hippocampus were not accompanied by changes in mRNA levels, as revealed by the absence of statistical significance when data were assessed by one-way analysis of variance (CA1: $F(4,32) = 1.182$, ns; CA2: $F(4,32) = 0.824$, ns; CA3: $F(4,32) = 0.581$, ns; CA4: $F(4,32) = 1.279$, ns; dentate gyrus: $F(4,32) = 0.532$, ns) (see Fig. 5).

Limbic structures

Specific binding

As occurred with the basal ganglia and the hippocampus, the daily administration of Δ^9-THC also produced a marked decrease of cannabinoid receptor binding in the septum nuclei ($F(4,35) = 3.529$, $P < 0.05$) and was almost statistically significant in the nucleus accumbens ($F(4,35) = 2.454$, $P = 0.064$), whereas a certain trend to decrease could be observed in the basolateral amygdaloid nucleus ($F(4,35) = 1.903$, $P = 0.132$). The post-hoc analysis revealed that the decrease in cannabinoid receptor binding already appeared 24 hours after the first Δ^9-THC injection in the nucleus accumbens (-15.8%), it was of similar magnitude after 3 days (-16.1%) and 7 days (-14.4%) of daily Δ^9-THC treatment, whereas it reached a maximum after 14 days (- 26.1%) (Fig. 6). The decrease in the septum nuclei, although it was not statistically significant 24 hours after the first Δ^9-THC injection (-10.2%), was progressively stressed afterwards (3 days: - 18.6%; 7 days: -24.1%; 14 days: -25.9%) (see Fig. 6). In contrast with these two limbic structures, specific binding in the basolateral amygdaloid nucleus did not change after 1 or 3 days of daily Δ^9-THC treatment, but a certain trend to decrease could be appreciated after 7 and 14 days (-18.0% and

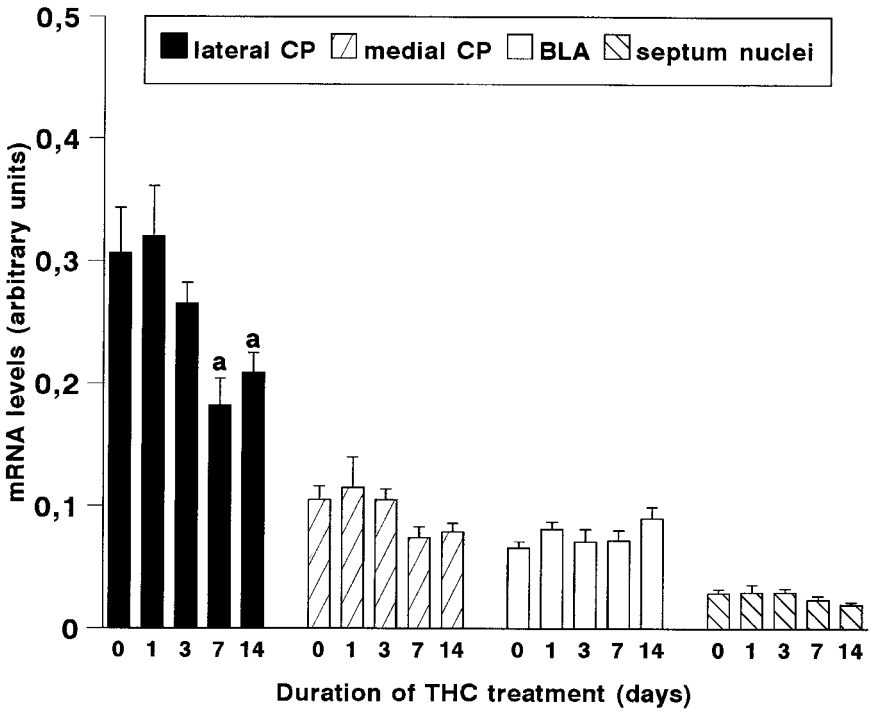

Fig. 3. Cannabinoid receptor mRNA levels (arbitrary units) measured by in situ hybridization in the lateral and medial caudate-putamen (CP), septum nuclei, and basolateral amygdaloid nucleus (BLA) of adult male rats daily exposed to Δ^9- tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls (0) were injected 7 days with vehicle. Details are in the text. Values are means ± SEM of 8–9 determinations *per* group. Data were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test (a: $P < 0.05$).

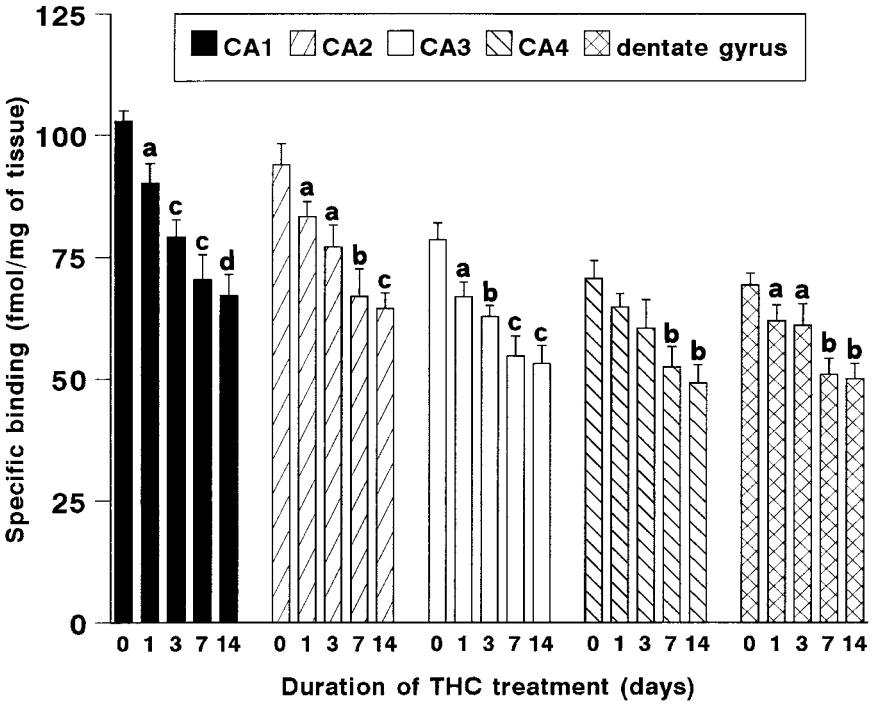

Fig. 4. Specific binding (fmol/mg tissue) for cannabinoid receptors measured by autoradiography in the Ammon's horn (CA1, CA2, CA3, and CA4 subfields) and dentate gyrus of the hippocampus of adult male rats daily exposed to Δ^9- tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls (0) were injected 7 days with vehicle. Details are in the text. Values are means ± SEM of 8–9 determinations *per* group. Data were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test (a: $P < 0.05$; b: $P < 0.005$; c: $P < 0.0005$; d: $P < 0.00005$).

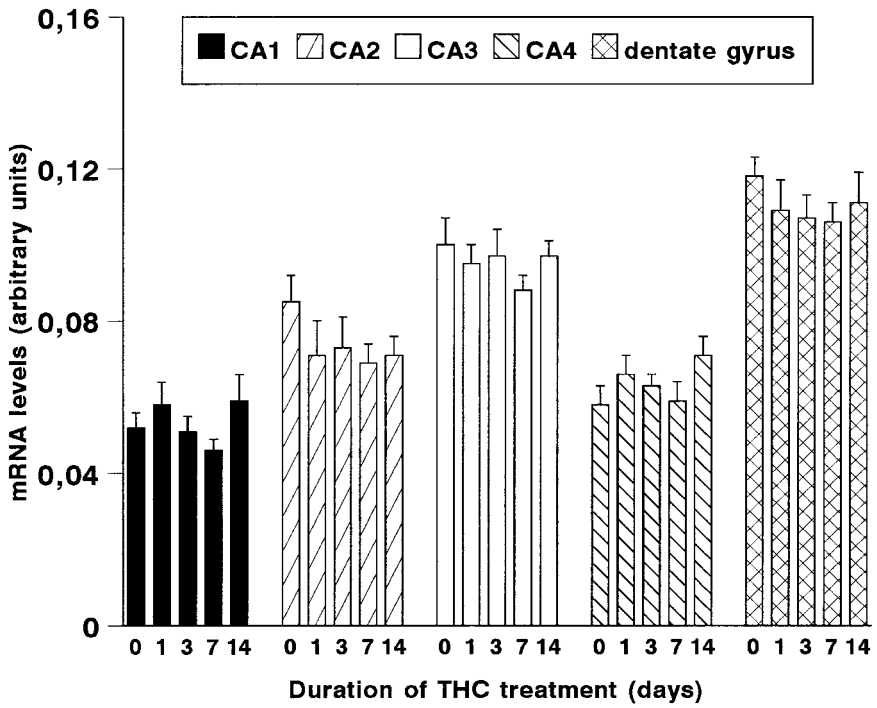

Fig. 5. Cannabinoid receptor mRNA levels (arbitrary units) measured by in situ hybridization in the Ammon's horn (CA1, CA2, CA3, and CA4 subfields) and dentate gyrus of the hippocampus of adult male rats daily exposed to Δ^9- tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls (0) were injected 7 days with vehicle. Details are in the text. Values are means ± SEM of 8–9 determinations *per* group. Data were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test.

-24.2%, respectively), although without reaching statistical significance (Fig. 6).

mRNA levels

As occurred in the hippocampal structures, the changes observed in cannabinoid receptor binding in the septum nuclei were not accompanied by changes in mRNA levels, as revealed the absence of statistical significance when data were assessed by one-way analysis of variance ($F(4,32) = 1.514$, ns) (Fig. 3). No changes were observed either in mRNA levels in the basolateral amygdaloid nucleus ($F(4,34) = 1.503$, ns) (Fig. 3).

Cerebral cortex

Specific binding

As in the above structures, the daily administration of Δ^9-THC also produced a marked decrease of cannabinoid receptor binding in the superficial ($F(4,33) = 3.684$, $P < 0.05$) and deep ($F(4,34) = 7.055$, $P < 0.0005$) layers of the cerebral cortex. The post-hoc analysis revealed that decrease in cannabinoid receptor binding already appeared 24 hours after the first Δ^9-THC injection in the deep layer (-10.6%), being progressively more marked after 3 days (-17.7%), 7 days (-26.9%), and 14 days (-32.0%) of daily Δ^9-THC treatment (Fig. 7). In the superficial layer, the decrease was not statistically significant after 1 and 3 days (-2.3% and -8.1%, respectively), but it was stressed after 7 days (-19.6%) and, particularly, 14 days (-27.0%) of daily Δ^9-THC treatment (Fig. 7). Representative autoradiograms used for the quantification of specific binding in the cortical structures of Δ^9-THC-exposed and control rats can be seen in Figure 2.

mRNA levels

As occurred in the hippocampal and limbic structures, the changes observed in cannabinoid receptor binding in the cerebral cortex were not accompanied by changes in mRNA levels, as revealed the absence of statistical significance when data were assessed by one-way analysis of variance (II–III layer: $F(4,32) = 0.75$, ns; V–VI layer: $F(4,32) = 2.026$, $P = 0.114$). Anyway, a certain trend could be observed in the deep layer with decreases after 7 and 14 days of daily Δ^9-THC treatment but they did not get statistical significance (Fig. 8). Representative autoradiograms used for the quantification of mRNA levels in the cortical structures of Δ^9-THC-exposed and control rats can be seen in Figure 2.

Hypothalamic structures

Specific binding

The daily administration of Δ^9-THC did not elicit any significant decrease of cannabinoid receptor binding in the ventromedial hypothalamic nucleus ($F(4,27) = 0.652$, ns) (Fig. 7).

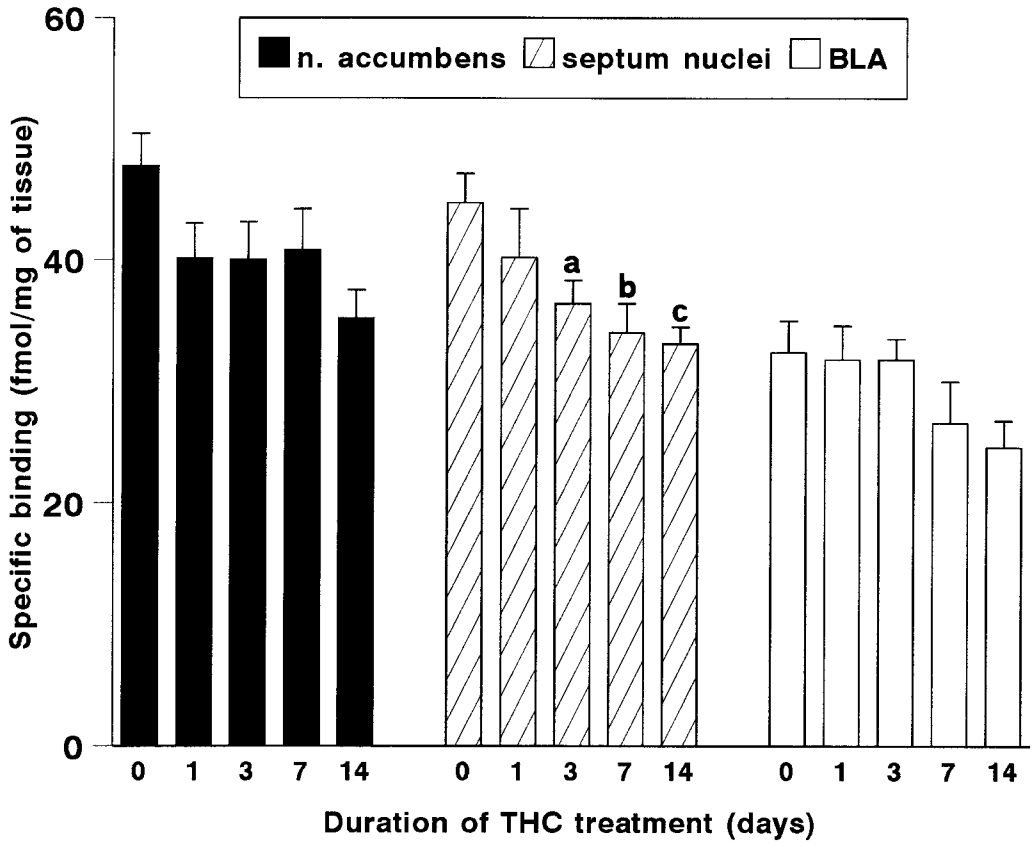

Fig. 6. Specific binding (fmol/mg tissue) for cannabinoid receptors measured by autoradiography in the nucleus accumbens, septum nuclei, and basolateral amygdaloid nucleus (BLA) of adult male rats daily exposed to Δ^9- tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls (0) were injected 7 days with vehicle. Details are in the text. Values are means ± SEM of 8–9 determinations *per* group. Data were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test (a: $P < 0.05$; b: $P < 0.005$; c: $P < 0.0005$).

mRNA levels

The absence of changes in binding in the ventromedial hypothalamic nucleus after several days of daily Δ^9-THC administration was accompanied by no changes in mRNA expression, as revealed by the absence of statistical significance when data were assessed by one-way analysis of variance ($F(4,35) = 0.96$, ns) (Fig. 8).

DISCUSSION

The results obtained in this study demonstrate again that the chronic activation of cannabinoid receptors results in a decrease in receptor binding in most of the brain structures examined. Only two areas, the entopeduncular nucleus and the ventromedial hypothalamic nucleus, exhibited no changes in cannabinoid receptor binding in concordance with previous results (de Miguel et al., 1998; Romero et al., 1997). Another two regions, the basolateral amygdaloid nucleus and the medial caudate-putamen, only exhibited non-statistically significant trends to decrease in binding, that were also in concordance with the small decreases observed in earlier reports (Rodríguez de Fonseca et al., 1994; Romero et al., 1997). The absence of changes in the ventromedial hypothalamic nucleus is particularly relevant because the process influenced by the activation of cannabinoid receptors in this area, i.e., the control of anterior pituitary hormone release, is one of the effects that did not exhibit tolerance after prolonged cannabinoid exposure (de Miguel et al., 1998).

With the only exception of these four areas, cannabinoid receptor binding decreased significantly and progressively after the daily Δ^9-THC administration in the remaining areas studied. These included basal ganglia (lateral caudate-putamen, substantia nigra, and globus pallidus), the hippocampus, limbic nuclei (nucleus accumbens and septum nuclei) and cerebral cortex. The magnitude of the decrease in binding was approximately similar to that obtained in previous studies (Oviedo et al., 1993; Rodríguez de Fonseca et al., 1994; Romero et al., 1995, 1997), around 30%. There were, however, some areas where the decrease was smaller, around 20%, as the basal ganglia, and, in particular, the globus pallidus (lesser than 10%). This particularity of the cannabinoid receptors in the basal ganglia has been extensively discussed in earlier reports (Romero et al., 1997) and seems to be related to the fact that there

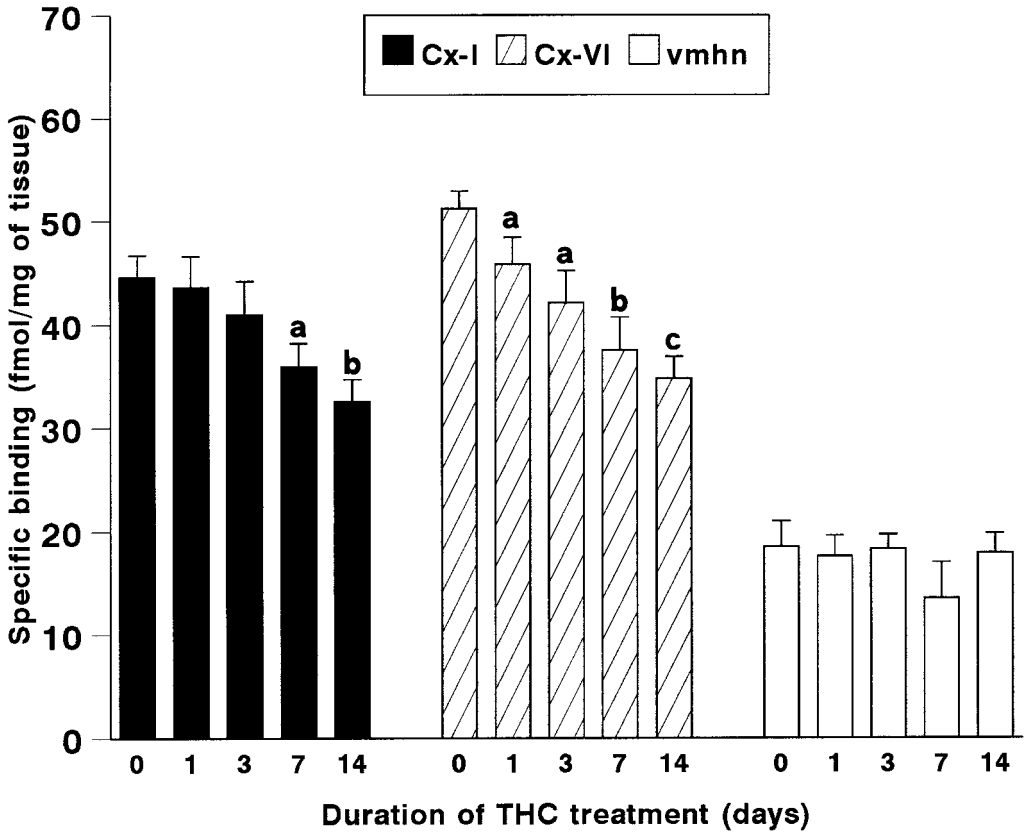

Fig. 7. Specific binding (fmol/mg tissue) for cannabinoid receptors measured by autoradiography in the superficial (Cx-I) and deep (Cx-VI) layers of cerebral cortex and in the ventromedial hypothalamic nucleus (vmhn) of adult male rats daily exposed to Δ^9-tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls (0) were injected 7 days with vehicle. Details are in the text. Values are means ± SEM of 8–9 determinations *per* group. Data were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test (a: $P < 0.05$; b: $P < 0.005$; c: $P < 0.0005$).

would be significant differences in cannabinoid receptors located in the three different striatal output neurons: striatonigral, striatopallidal, and striatoentopeduncular. These differences would be not only vs. cannabinoid receptors in the non-extrapyramidal regions, but also among the three different striatal pathways. Thus, in the present study, we have observed that the population of cannabinoid receptors in striatonigral neurons moderately responded to chronic Δ^9-THC exposure, whereas the response was very small in striatopallidal neurons and did not exist in striatoentopeduncular ones. This variation led to an also moderate response of cannabinoid receptor binding in the lateral part and only a trend in the medial part of the caudate-putamen, the regions where cell bodies of these three pathways are located.

In addition to these regional differences in the magnitude of cannabinoid receptor down-regulation, another important aspect provided by the present results is the elucidation of the onset of this process, which also presented significant regional differences. Thus, the decrease was very rapid in the hippocampal structures (most of the subfields of the Ammon's horn and the dentate gyrus) and in the deep layer of the cerebral cortex, since it already appeared 24 hours after the first Δ^9-THC administration. Other areas, such as the caudate-putamen, substantia nigra, and septum nuclei, needed at least 3 days of daily Δ^9-THC administration, whereas others, such as the globus pallidus and the superficial layer of the cerebral cortex, were particularly resistant to chronic Δ^9-THC-induced down-regulation, needing more than 3 days of Δ^9-THC administration. These regional differences are concordant with the results obtained by Breivogel et al. (1997), using WIN-55,212–2-stimulated [^{35}S]-GTPγS binding, which is indicative of receptor activation of signal transduction mechanisms. These authors found that desensitization occurred slowly in the globus pallidus, whereas the effect was more rapid in the hippocampus, in both cases as occurred in our present study analyzing receptor binding.

The third aspect of our present results that deserves to be mentioned deals with the parallelism between changes in receptor binding and the potential variations in mRNA expression. According to our present results, it can be asserted that the changes of binding

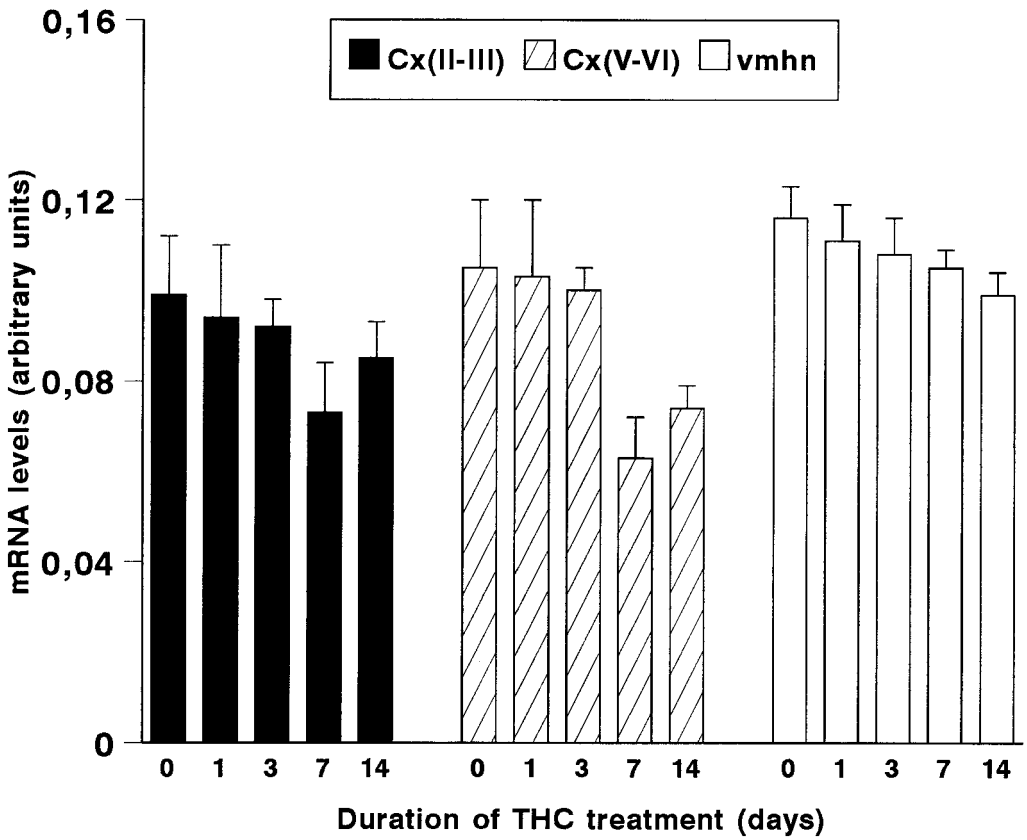

Fig. 8. Cannabinoid receptor mRNA levels (arbitrary units) measured by in situ hybridization in the superficial (Cx-II-III) and deep (Cx-V-VI) layers of cerebral cortex and in the ventromedial hypothalamic nucleus (vmhn) of adult male rats daily exposed to Δ^9-tetrahydrocannabinol (Δ^9-THC) during 1, 3, 7, or 14 days. Controls (0) were injected 7 days with vehicle. Details are in the text. Values are means ± SEM of 8–9 determinations *per* group. Data were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test.

precede the changes in mRNA expression, when these appeared as the case of the caudate-putamen and certain trends in the cerebral cortex. Thus, the reduction in binding in the lateral caudate-putamen appeared at 3 days of daily Δ^9-THC administration, whereas the decrease in mRNA levels in this area needed more than 3 days. In other brain structures, changes in mRNA levels did not exist, which coincides with that reported by Rubino et al. (1994), who found changes only in the caudate-putamen, using in situ hybridization, and by us (Romero et al., 1997), using Northern blot analysis.

In summary, the daily administration of Δ^9-THC resulted in a progressive decrease in cannabinoid receptor binding in most of the brain areas studied, which always preceded the changes in mRNA expression in those areas where these existed. The onset of the decrease in receptor binding exhibited regional differences with areas, such as most of the hippocampal structures and the deep layer of the cerebral cortex, where the decrease occurred after the first administration. Other structures, however, needed at least 3 days or more to initiate receptor binding decrease. Two structures, the entopeduncular nucleus and the ventromedial hypothalamic nucleus, were unresponsive to chronic Δ^9-THC administration, whereas others, such as the medial caudate-putamen and the basolateral amygdaloid nucleus, only exhibited trends.

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