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Chronic Δ-9-tetrahydrocannabinol treatment increases cAMP levels and cAMP-dependent protein kinase activity in some rat brain regions

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Abstract

When Δ^9-tetrahydrocannabinol (Δ^9-THC,15 mg/kg) was injected intraperitoneally twice a day for 6 days, tolerance to its analgesic effect appeared to be complete. Chronic exposure to Δ^9-THC caused a significant reduction in CB1 receptor binding in all brain areas that contain this receptor. Cannabinoid receptor density was markedly reduced in the cerebellum (52%), hippocampus (40%) and globus pallidum (47%) compared to 30% in the cortex and striatum. Chronic exposure enhanced the cAMP pathway, as shown by the significant increase of cAMP levels and PKA activity in the areas with receptor down-regulation (cerebellum, striatum and cortex). We propose that the increase in cAMP cascade is part of the biochemical basis of cannabinoid tolerance.

Keywords: Δ^9-Tetrahydrocannabinol; Chronic treatment; Central nervous system; cAMP accumulation; PKA activity

1. Introduction

Several studies have attempted to correlate cannabinoid behavioural tolerance with biochemical alteration, and this tolerance appears to be both pharmacokinetic and pharmacodynamic in nature. Chronic treatment with CP-55,940 increased the activity of the microsomal cytochrome P450 oxidative system (Costa et al., 1996), suggesting some pharmacokinetic tolerance. Chronic cannabinoid treatments also changed brain cannabinoid receptor mRNA levels (Abood et al., 1993; Rubino et al., 1994; Romero et al., 1997; Zhuang et al., 1998) depending on the time course of treatment and differing in various brain regions, indicating that pharmacodynamic effects were important as well. Brain cannabinoid receptor levels usually dropped after prolonged exposure to agonists (Oviedo et al., 1993; Rodriguez de Fonseca et al., 1994; Fan et al., 1996; Romero et al. 1997, 1998) although some studies reported increases (Romero et al., 1995) or no change in receptor binding in the brain.

Chronic treatment with Δ^9-THC also affects cannabinoid-mediated signal transduction systems. Fan et al. (1996) examining the effect of in vivo chronic CP-55,940 treatment, found no changes in adenylyl cyclase in cerebellar membranes despite a 50% reduction in receptor binding, suggesting some receptor reserve. Chronic Δ^9-THC induced a steep drop in WIN55212-2-stimulated [^{35}S]GTPγS binding after 21 days treatment, the reduction ranging from 40% to 80% in agonist-induced labelling (Sim et al., 1996). The desensitization produced by Δ^9-THC varied in its time course across different rat brain regions, suggesting that the time course of Δ^9-THC induced tolerance may differ for the various different effects of cannabinoids. Finally, our previous work (Rubino et al., 1997) indicated that the expression of G-protein α-subunit mRNAs in rat brain was significantly altered by in vivo chronic CP-55,940, suggesting that this change might be a molecular event associated with the development of cannabinoid tolerance.

In spite of these results, in vivo studies of the cellular

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changes responsible for the adaptive modifications underlying the development of cannabinoid behavioural tolerance are still young. All evidence points to the brain cannabinoid receptors being negatively coupled to adenylyl cyclase, so cellular desensitization should logically be a necessary part of the development of tolerance. This was confirmed in cultured neuroblastoma cells where chronic cannabinoid exposure desensitized cannabinoid-inhibited adenylyl cyclase (Dill and Howlett, 1988), indicating that cannabinoid tolerance may involve alterations in signal transduction. The aim of the present study was to determine the alterations in the cAMP cascade in specific brain regions taken from rats tolerant to Δ^9-THC, in terms of cAMP accumulation and PKA activity induced by in vivo treatment.

2. Methods

2.1. Animals and treatment

Male Sprague–Dawley rats (Charles River, Calco, Italy) weighing 150–175 g at the beginning of the experiment were used. The animals were housed three per cage in standard conditions. For the acute treatment rats received Δ^9-THC (a generous gift from the National Institute of Drug Abuse, USA) intraperitoneally (i.p.) at a dose of 15 mg/kg, or its vehicle (ethanol, cremophor and saline 1:1:18). To induce tolerance Δ^9-THC was injected twice a day (between 9:00 and 10:00 a.m. and 5:00 and 6:00 p.m.) for 6.5 days at a dose of 15 mg/kg i.p. Control animals were given the vehicle at the same times. On days 1, 4 and 6 the analgesic effect of Δ^9-THC was evaluated by the tail flick test to monitor the development of tolerance. Pain thresholds were evaluated 15, 30, 60 and 120 min after the morning dose. A maximum cut-off time of 15 s was used and the rat was removed from the apparatus if it failed to respond within this interval. The results were expressed as total area under the time–response curve (AUC) over the 120 min.

2.2. Biochemical studies

2.2.1. cAMP level

One hour after the Δ^9-THC injection in either acute or chronic conditions, the rat brains were quickly removed and the various areas (cortex, striatum, mesencephalon and cerebellum) were obtained by gross dissection on ice, according to the atlas of Paxinos and Watson (1986). Tissues were homogenized in ice-cold buffer containing 0.25 M sucrose, 50 mM Tris–HCl pH 7.5, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 0.1 mM DTT, 0.1% Triton X100, 10 μg/ml leupeptin, 10 μg/ml aprotinin. To measure cAMP level, 15 μl were diluted 1:15 with 50 mM Tris–HCl, pH 7.5, 5 mM EDTA and boiled for 5 min to coagulate protein. After 5 min centrifugation at 1200 ***g***, the cyclic AMP in the supernatant was assayed using a TRK 432 radioreceptor assay kit (Amersham, Milan, Italy). Protein was assayed by the method described by Bradford (1976).

2.2.2. PKA activity

PKA was assayed as previously described (Koh et al., 1997), with some modifications. In brief, the isolated brain regions were homogenized as for cAMP assay; lysates were centrifuged at 270 ***g*** for 2 min and the supernatant was used as cellular extract. The reaction mixture of 40 μl contained 10 μl of cellular extract, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 100 μM ATP (20 μCi/ml [γ-[^{32}P]ATP]), 0.25 mg/ml bovine serum albumin, and 50 μM Kemptide as PKA substrate (the heptapeptide Leu–Arg–Arg–Ala–Ser–Leu–Gly). The background level of each group was measured in the presence of 1 μM PKI(6-22)amide, and total activity was measured in the presence of 10 μM exogenous cAMP. Samples were incubated at 37°C for 10 min. Phosphocellulose disks were then spotted with 20 μl of sample, followed by two acid washes [1% (v/v) phosphoric acid] and two water washes. ^{32}P was quantified by scintillation counting.

2.2.3. Autoradiographic binding

Δ^9-THC treated animals were killed 1 h after the last injection and their brains were rapidly removed and frozen by being gently lowered into liquid nitrogen. They were stored at −80°C. For processing, brains were brought to −16°C in a cryostat and a series of 12 μm-thick serial sections was collected on gelatin-coated slides. The sections were briefly dried at 30°C and stored at −80°C until they were processed for receptor binding autoradiography.

Binding was done as described previously (Rubino et al., 1997). The slides were brought briefly to room temperature, then incubated for 2.5 h at 37°C with 10 nM [^{3}H]CP-55,940 (NEN, custom-labeled, 129.1 Ci/mmol) in binding buffer (50 mM Tris–HCl, pH 7.4, 5% BSA). Adjacent brain sections were incubated in parallel with 10 μM CP-55,940 to assess non-specific binding. Sections were washed twice for 4 h at 0°C in 50 mM Tris–HCl (pH 7.4) and 1% BSA buffer. The sections were then dipped briefly in water to remove excess BSA and dried under a cool stream of air. Autoradiograms were made by exposing the dried sections in X-ray cassettes to tritium-sensitive film Hyperfilm-^{3}H (Amersham, Milan, Italy) for 10 days. Autoradiograms were developed with Kodak D 19 developer (25°C, 4 min), fixed in Kodak Unifix (8 min) and rinsed with water (15 min).

The intensity of the receptor binding signal was assessed by measuring the grey levels of the autoradiographic films with an image analysis system consisting of a solid-state video camera (Hamamatsu, Japan) connected to an Apple Macintosh II personal computer. The public domain Image 1.47 software was used (NIH,

Bethesda, MD, USA). Each brain section was traced with mouse cursor control and the light transmittance was determined as grey level. The grey level of densitometric measurements calculated after subtraction of the film background density was established within the linear range, determined using tritium standards ([^{3}H]Microscales, Amersham, Milan, Italy).

2.2.4. Statistical analysis

Data are the mean±SEM of at least six rats. The significance was established with one-way analysis of variance (ANOVA) followed by Tukey's test, except for autoradiographic binding studies where Student's *t*-test was used.

3. Results

3.1. Behavioural studies

The effect of repeated Δ^9-THC injections (15 mg/kg i.p./twice a day for 6 days) on analgesia is reported in Fig. 1. The first injection produced a significant degree of analgesia as indicated by the marked increase in the AUC in acutely injected rats vs control. Four days exposure to Δ^9-THC significantly reduced the AUC by about 50% vs first injection (data not shown) while within 6 days, tolerance appeared to be complete, the area under the time–response curve giving the same value as controls.

3.2. Autoradiographic receptor binding

Six days exposure to Δ^9-THC significantly reduced specific binding to CB1 receptors in most of the brain regions studied (Fig. 2). The most sensitive regions were the cerebellum (107±3.7 fmol/mg tissue in control vs 55.4±5.2 fmol/mg tissue in tolerant), hippocampus (101.6±1.9 fmol/mg tissue vs 62.8±5.8 fmol/mg tissue), and globus pallidum (157.2±6.6 fmol/mg tissue vs 82.2±6.4 fmol/mg tissue). The reduction in the cortex and striatum reached only 30% (cortex 57.3±3.3 vs 40.1±1.4 fmol/mg tissue and striatum 101±3.2 vs 73.9±1.01 fmol/mg tissue), confirming regional differences in the magnitude of the receptor down-regulation. As expected, the changes seen in specific binding of CB1 receptors after chronic exposure to Δ^9-THC, did not exist when the cannabinoid was given acutely, ruling out any interference from the drug pre-bound to the receptor in binding kinetics (data not shown).

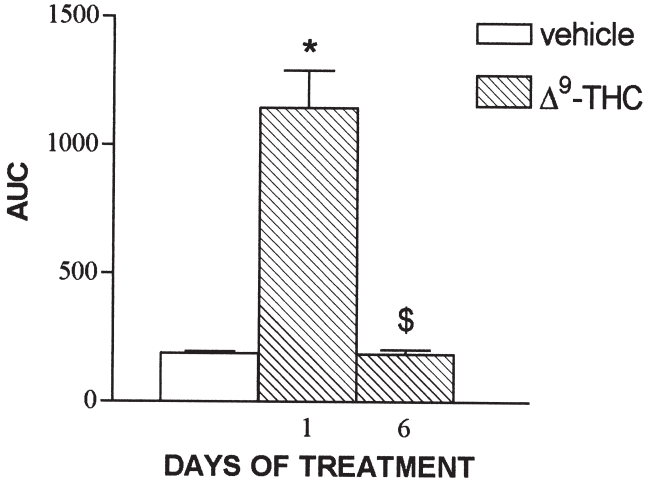

Fig. 1. Effect of Δ^9-THC (15 mg/kg i.p. twice daily for 6.5 days) on analgesia expressed as area under the analgesic curve (AUC). The data are the mean±SEM of at least 8 animals. *$p<0.001$ vs vehicle; $^{\$}p<0.001$ vs first day.

3.3. Chronic effects of Δ^9-THC on cAMP production and PKA activity

One hour after the last Δ^9-THC injection on day 7, the rats were killed and cellular cAMP levels and PKA activity were determinated in some brain regions of tolerant and control rats. In view of the abundance of cannabinoid receptors, the initial assays were done in the cerebellum, striatum and cortex. The mesencephalon was chosen as a negative area. As shown in Fig. 3, long-term exposure to the cannabinoid agonist, but not acute dosing, significantly raised cAMP levels at the cortex (66% increase), striatum (75%) and cerebellum (108%). cAMP accumulation in the mesencephalon did not change. As shown in Fig. 4, the brain areas of tolerant rats also showed an alteration in PKA activity that seemed to parallel that observed for cAMP. This activity was increased in the areas also positive for cAMP, namely the cortex (50%), striatum (20%) and cerebellum (24%) while the mesencephalon appeared unresponsive.

4. Discussion

These findings indicate that chronic treatment of rats with Δ^9-THC resulted in the development of full tolerance to its analgesic effect, with a significant decrease in receptor binding in all the brain structures examined. The most important areas containing CB1 receptors, namely the striatum, the cortex, the hippocampus and cerebellum, presented a reduction greater than 38%, confirming previous results from other groups (Oviedo et al., 1993; Rodriguez de Fonseca et al., 1994; Romero et al., 1998) although with some differences in magnitude. The greater reduction in our study might be attributable to our protocol which involved repeated, fairly large doses of Δ^9-THC (15 mg/kg i.p./twice a day). The degree of tolerance depends on both the intensity and the length of treatment, which can cause different or more sustained adaptive events to arise. Our widespread decrease

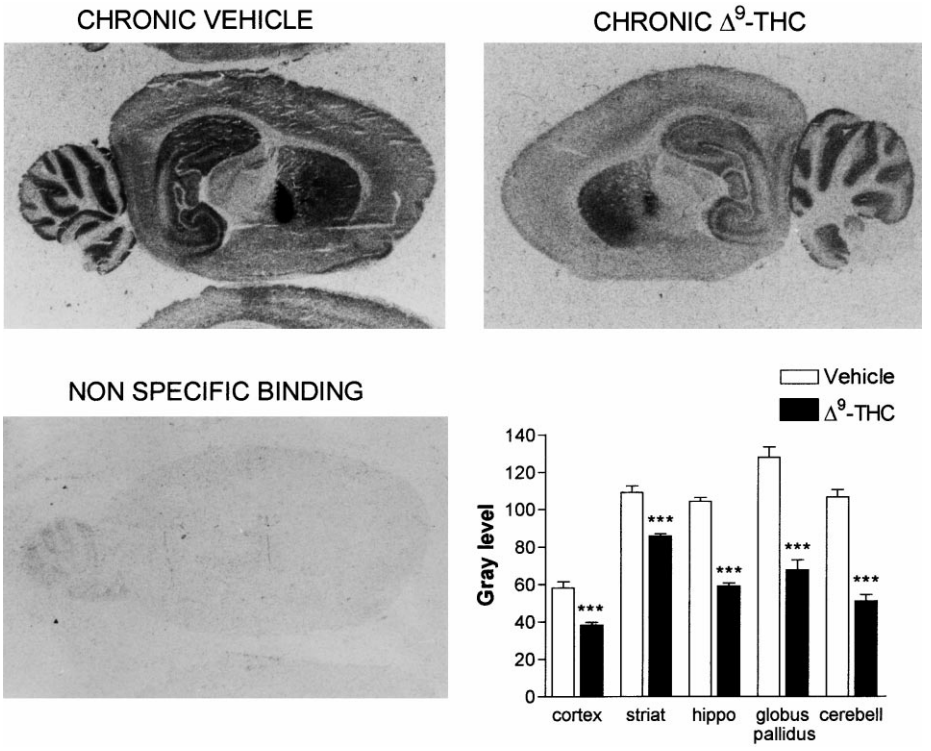

Fig. 2. Autoradiograms of representative sagittal sections of the rat brain showing the effect of chronic Δ^9-THC (15 mg/kg i.p. twice a day for 6.5 days) on CB1 receptor density. The bottom right panel shows the quantitation of CB1 receptor density in the cerebral areas presented in autoradiograms. ***$p<0.0001$ vs vehicle (Student's *t*-test).

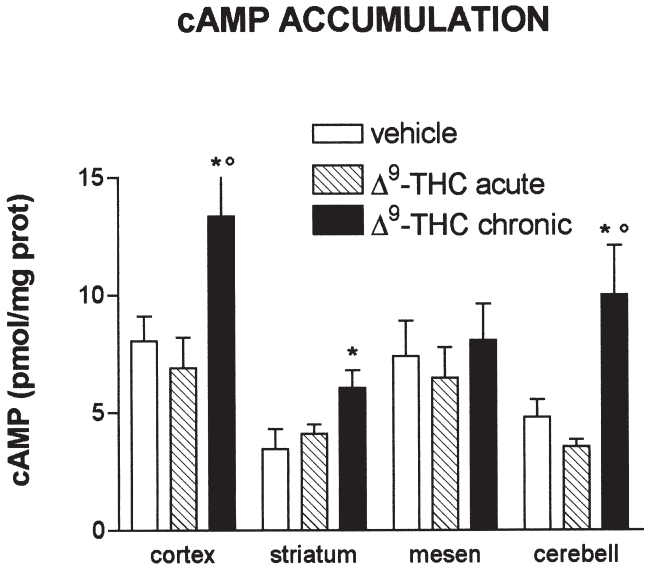

Fig. 3. Effect of in vivo treatment with Δ^9-THC injected acutely (15 mg/kg i.p) or chronically (15 mg/kg i.p. twice a day for 6.5 days) on cAMP levels in different brain areas. The data are the mean±SEM of at least 6 animals. *$p<0.05$ vs vehicle; °$p<0.05$ vs acute Δ^9-THC (Tukey's test).

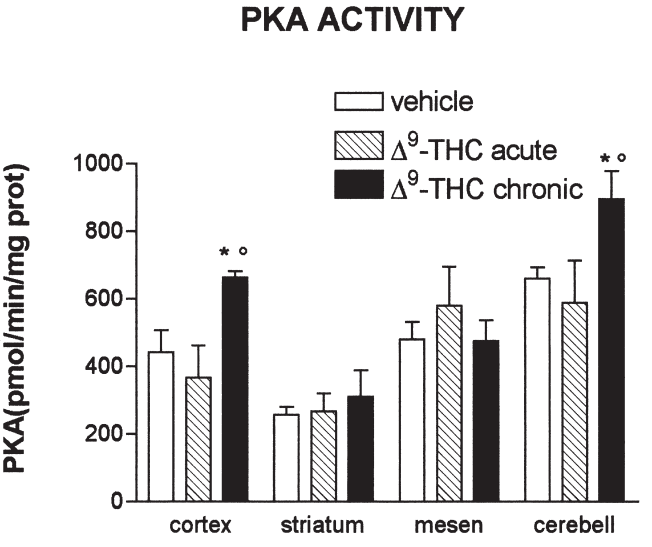

Fig. 4. Effect of chronic in vivo treatment with Δ^9-THC injected acutely (15 mg/kg i.p) or chronically (15 mg/kg i.p. twice a day for 6.5 days) on PKA activity in different brain areas. The data are the mean±SEM of at least 6 animals. *$p<0.05$ vs vehicle; °$p<0.05$ vs acute Δ^9-THC (Tukey's test).

in CB1 binding together with the cellular adaptations we found on cAMP and PKA activity could justify the complete tolerance to cannabinoid induced analgesia. However, our data further confirm that receptor down-regulation is one of the processes involved in cannabinoid tolerance.

Another important point we set out to clarify was whether the desensitization of the cAMP system paralleled the appearance of receptor down-regulation in

chronically-treated rats. In our hands, long-term exposure of rats to Δ^9-THC led to an increase in the level of cAMP and to a closely related increases in PKA detected in the same brain regions, i.e. the cerebellum, the cortex and the striatum. The most obvious explanation for this result could be an increase in adenylyl cyclase activity. In this regard, Fan et al. (1996) examining the effect of in vivo treatment with CP-55,940, found any alteration in adenylyl cyclase activity in the cerebellum of tolerant mice, despite a strong reduction in cannabinoid receptor density. More recently, Hutcheson et al. (1998) demonstrated that SR141716A-precipitated withdrawal in mice was expressed, at the cellular level, by a pronounced increase of adenylyl cyclase activity that was selectively observed in the cerebellum. Moreover in cultured neuroblastoma cells (Dill and Howlett, 1988; Shapira et al., 1988), chronic cannabinoid exposure desensitized cannabinoid inhibited adenylyl cyclase. However the assay we used (cAMP content) does not allow us to exclude the possibility that the cAMP increase could be linked to an inhibition in phosphodiesterase activity although evidence on the influence of cannabinoid on these enzymes is lacking. Finally, another intriguing explanation for the cAMP increase is the possibility that the chronic treatment could unmask an alternate CB1 receptor-mediated signalling pathway involving the stimulation of adenylyl cyclase through a Gs linkage, as already suggested by Glass and Felder (1997) in primary striatal cultures. Although the natural basal constitutive activity of the cannabinoid CB1 receptor normally manifests itself through an inhibition of adenylyl cyclase activity, there may be certain situations (in our hands Δ^9-THC chronic treatment) where the alternative Gs coupling appears, probably through an imbalance in the neuron G protein components, as already hypothesized by Calandra et al. (1999).

Furthermore it appears that in the regions with the highest CB1 receptor density there is a negative feedback response to the action of the chronic cannabinoid. Acutely, cannabinoids inhibit adenylate cyclase activity not only in cultured neuroblastoma cells, but also in membranes prepared from different brain regions (Bidaut-Russel et al., 1990; Pacheco et al., 1994), and this inhibition would be expected to lead to lower levels of cyclic AMP and of activated cyclic AMP-dependent protein kinase. In our hands acute in vivo Δ^9-THC provoked only a slight reduction in these biochemical parameters, as already demonstrated under the same conditions for opioids (Nestler and Tallman, 1987) and expected when the assays are performed without an extra in vitro treatment with the drug. However, the fact that the biochemical parameters both increased in response to chronic cannabinoid treatment may indicate an attempt by several brain areas to compensate for and overcome this chronic decrease in cAMP system produced by Δ^9-THC.

Another important aspect is the region-specific nature of the chronic effect of Δ^9-THC. In fact the cAMP cascade was altered only in areas containing the CB1 receptor, but not, for instance, in the mesencephalon where there are very few cannabinoid receptors. This adaptive event may, therefore, be closely related to the cannabinoid receptor stimulation and is not a receptor-independent effect. Experiments are now in progress to verify whether the globus pallidus and the hippocampus, two other important regions rich in CB1 receptors, present similar adaptive responses.

However, our in vivo data agree with the rebound effect observed in N18TG2 neuroblastoma cells chronically exposed to the cannabinoid agonist desacetyllevonantradol (DALN) that presented an elevated basal cAMP level as well an elevated basal G protein activity (Shapira et al., 1988). In contrast Sim et al. (1996) reported that in rats, in vivo treatment with Δ^9-THC (10 mg/kg i.p. 21 days) significantly reduced the net WIN 55212-2 stimulated [^{35}S]GTPγS binding in most brain regions including the caudate putamen, perirhinal and entorhinal cortex, globus pallidus and cerebellum, suggesting that there is marked desensitization of cannabinoid-activated signal transduction mechanisms after chronic Δ^9-THC treatment. GTPγS binding in chronic rats only increased in the cerebellum (20%), in the other brain regions considered, no difference was noted between control and chronic-treated rats. Once again the different brain regions present different adaptive responses to chronic Δ^9-THC. To assess our data together with those of Sim et al. (1996), we can assume that the desensitization of the cannabinoid activated signal transduction mechanisms induced by Δ^9-THC causes an absence of the inhibitory tone in discrete brain regions mediated through the cannabinoid receptor, resulting in up-regulation of the cAMP system. The time course may differ in the different brain areas with some being more sensitive and presenting rapid adaptation, and others needing longer.

Interestingly, the effect of chronic Δ^9-THC on the cAMP system resembles those of opioids. Nestler and Tallman (1987) reported that chronic but not acute in vivo treatment of rats with morphine increased cAMP-dependent protein kinase activity in the locus coeruleus, with a time course by which locus coeruleus neurones become tolerant to and dependent on opiates. There is a fundamental difference between Nestler's results and ours. The changes in the cAMP system in response to chronic morphine showed a region-specific nature, being present only in the locus coeruleus and absent in other brain regions, such as the neostriatal frontal cortex and dorsal raphe, despite the presence of opioid receptors. With chronic Δ^9-THC there was a widespread increase in the cAMP system throughout all the brain regions expressing CB1 receptors. The same difference was found in the GTPγS assay, the changes induced by

chronic morphine being small and anatomically discrete and those of chronic Δ^9-THC large and widespread. As already suggested by Sim et al. (1996), the well-known difference in catalytic efficiency of these two receptors may have consequences during chronic treatment resulting in greater adaptive changes in receptor-G protein coupling in the less efficiently coupled system.

In summary these findings indicate that during chronic exposure, Δ^9-THC regulates the cyclic AMP system in discrete brain regions expressing CB1 receptors. We propose that the up-regulation of this system at several levels, which would be expected to increase neuronal excitability, may be part of the biochemical basis of cannabinoid addiction.

Acknowledgements

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