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Pharmacology, Biochemistry and Behavior 81 (2005) 300 – 318

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Review

Cannabinoid tolerance and dependence: A review of studies in laboratory animals

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Received 19 December 2004; received in revised form 18 January 2005; accepted 18 January 2005
Available online 24 May 2005

Abstract

Two are the issues on cannabis addiction that provoke more controversy from a research perspective. The first one is related to the development of tolerance phenomena and, in particular, of a dependence state after chronic cannabinoid consumption, with appearance of withdrawal signs when this is interrupted, that would be (or not) comparable to those observed for other drugs. A second controversial issue is related to the possibility that chronic cannabinoid consumption may increase the risk to consume other drugs of greater addictive power. Since the discovery in the 1990s of the endocannabinoid signaling system as the target for the action of plant-derived cannabinoids, many studies have addressed these two questions in laboratory animals and, although the results have resulted controversial in various aspects, the following conclusions seem evident: (i) prolonged exposure to plant-derived, synthetic or endogenous cannabinoid agonists in laboratory animals is currently associated with the development of tolerance for most of their pharmacological effects, (ii) tolerance is essentially due to adaptative phenomena consisting in pharmacodynamic events (down-regulation/desensitization of cannabinoid receptors), although some evidence exist on additional pharmacokinetic responses, (iii) the discontinuation of chronic cannabinoid treatment does not elicit abstinence responses spontaneously in most of the cases, presumably because the pharmacokinetic characteristics of cannabinoids, but these responses may be elicited after the blockade of cannabinoid CB_1 receptors in cannabinoid-tolerant animals, (iv) these abstinent responses include mainly somatic signs and changes in various molecular processes affected during the abstinence to other drugs although the magnitude of these changes was currently lower in the case of cannabinoids, and (v) cannabinoid-tolerant animals do not appear to be more vulnerable to reinforcing properties of morphine, although the manipulation of the endocannabinoid signaling might serve to treat cannabis addiction and, in particular, the addiction to other drugs such as alcohol, nicotine or opioids. The present review article will address all these aspects trying to establish the bases for future research.

Keywords: Endocannabinoids; Cannabinoid CB_1 receptor; Tolerance; Dependence; Precipitated-withdrawal; Δ^9-tetrahydrocannabinol; SR141716

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doi:10.1016/j.pbb.2005.01.028

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1. Introduction

Cannabis sativa derivatives, such as marijuana and hashish, are among the drugs of abuse whose consumption has higher prevalence, particularly among young people (Budney et al., 1999; Ashton, 2001). Marijuana has always been considered as a "soft drug" in part due to its lower impact on human health compared to opioids, alcohol or psychostimulants, and in part due to the late discovery of mechanisms of action in the brain of its psychoactive constituents. Due to these two considerations, various aspects of cannabis addiction have resulted to recent controversies with some being clarified only very recently, among them: (i) whether cannabis is addictive as what happens with other recreational substances consumed by humans; (ii) whether a prolonged exposure to cannabinoids develops tolerance due to similar responses operating for other drugs, i.e., down-regulation/desensitization of specific receptors, changes in bioavailability, or neuronal adaptation; this point is also important considering the possible regular therapeutic use of cannabinoids in specific diseases; (iii) whether there exists an abstinence syndrome equivalent to that occurring in the case of other habit-forming drugs of recognized greater addictive power; and (iv) whether cannabis might play a gateway effect by increasing the risk to consume these more strongly addictive drugs. With the exception of the first of these four issues, which has been addressed in the review article written by Elliot Gardner in this special issue (Gardner, 2005), the present review will address all literature published, mainly in laboratory animals, on the remaining three issues of this purported addiction to cannabis.

2. Do cannabinoids produce tolerance?

The first contact(s) with cannabis in humans, which can be reproduced by acute or subchronic administrations of cannabis extracts or selected cannabinoids in laboratory animals, produce(s) a large spectrum of neurobiological effects, among them, euphoria followed by sedation, analgesia, motor inhibition, ataxia, incoordination, anticonvulsing activity, memory impairment, anxiety effects, antiemesis, hypothermia, increased appetite and other effects (Dewey, 1986; Hollister, 1986; Pertwee, 1995; Abood and Martin, 1992; Adams and Martin, 1996; Hampson and Deadwyler, 1999; Gardner, 2002). It is important to remark that, in laboratory animals, these data were obtained after directly administering cannabis or selected cannabinoids since, as reviewed by Justinova et al. (2005) in this special issue, drug self-administration, which would be the best way to reproduce in laboratory animals the pattern of drug consumption in humans, is not currently observed for cannabinoids in laboratory animals except in the case of certain agonists with particular pharmacokinetic characteristics or under certain experimental conditions. Cannabinoids also produce a variety of peripheral effects, such as hypotension, reduction of intraocular pressure, immunosuppression, intestinal hypomotility, and changes in adrenal function (Pertwee, 1991; Howlett et al., 2004). There is a general agreement that most of these central and peripheral effects of cannabinoids develop tolerance when the administration prolongs for several days in laboratory animals (Dewey, 1986; Abood and Martin, 1992; Maldonado and Rodríguez de Fonseca, 2002). Tolerance occurs in a wide range of species and has been also demonstrated in in vitro cell culture experiments (reviewed by Pertwee, 1991). These observations reproduce the situation in humans where a phenomenon of pharmacological tolerance has been reported for most of effects of cannabis (Jones et al., 1981; Hollister, 1986). However, the usual pattern of social cannabis use might not lead to tolerance, except in the case of extremely heavy social abusers or after the expected regular therapeutic uses in humans (Haney et al., 1999; Hart et al., 2002). On the other hand, another consequence of chronic cannabinoid exposure is the development of behavioral sensitization (Rubino et al., 2001, 2003; Cadoni et al., 2001), a phenomenon well-described for other drugs and that contributes to the increased drive and motivation for the substance (see Nestler, 2004, for review).

In laboratory animals, the degree and the time-course of tolerance are dependent on the species used, type of ligand, the dosage and the duration of the treatment, the measures employed to determine tolerance and the system in which it is assessed. As will be detailed below, the pharmacokinetic properties of cannabinoids (changes in drug absorption, distribution, biotransformation, and excretion) also influence the degree of tolerance, although their impact seems minor compared to the important role played by their

pharmacodynamic properties (see below). Thus, measures of tolerance for certain pharmacological effects of cannabinoids, such as analgesia, motor inhibition, hypothermia, are typically within the range of 3–7 days (Pertwee, 1991; Pertwee et al., 1993; Abood et al., 1993; Oviedo et al., 1993; Fan et al., 1994; Rubino et al., 1997; Bass and Martin, 2000), but other effects, such as memory effects (Deadwyler et al., 1995) or certain neuroendocrine actions (De Miguel et al., 1998; González et al., 1999), resulted to be extremely resistant needing weeks or months to develop. This also means that the neural substrates underlying the different brain functions affected by cannabinoids adapt differently to a prolonged cannabinoid administration, so that the rate and magnitude of the neuroadaptative processes resulting in functional tolerance may be significantly different (Rubino et al., 2000b).

As mentioned above, one relevant case concerns the memory effects of cannabinoids. There is an early study reporting lack of tolerance to the memory impairment caused by Δ^9-tetrahydrocannabinol (Δ^9-THC) in monkeys (Ferraro and Grilly, 1973), which is concordant with clinical observations in humans that memory impairment does not disappear or reduce in chronic marijuana users (Chait and Perry, 1994; Heishman et al., 1997). In addition, other studies showed that WIN55,212-2 (Hampson et al., 2003) and Δ^9-THC (Deadwyler et al., 1995) developed tolerance to their memory disruptive effects, although this takes several weeks. These authors related this phenomenon to adaptation of hippocampal neurons (see below). By contrast, other studies reported rapid tolerance to the amnesiac effects of cannabinoids (see Sim-Selley, 2003, for review), stating that differences in dosage and duration of chronic treatment may account for these discrepancies. Other cannabinoid effects are in the opposite side to their memory effects. This is the case of some effects in rodents, such as motor inhibition, hypothermia, analgesia, immobility, and anticonvulsant activity (Oviedo et al., 1993; Welch et al., 1995; Fan et al., 1996), as well as for the static ataxia observed in dogs (Martin et al., 1975), which easily developed tolerance phenomena for different cannabinoid agonists. These responses were found after Δ^9-THC but also after other analogs, such as Δ^8-THC, 11-hydroxy-Δ^9-THC and 11-hydroxy-Δ^8-THC (see Pertwee, 1991, for review), or synthetic cannabinoid agonists, such as CP-55,940, levonantradol, nabilone or WIN55,212-2 (Pertwee, 1991; Pertwee et al., 1993; Oviedo et al., 1993). However, even in the cases where tolerance developed more intensely, the magnitude and the time needed for this phenomenon to develop exhibited subtle differences possibly based on small differences in pharmacodynamic (receptor affinity or potency) and pharmacokinetic (metabolic stability, bioavailability) characteristics of the different cannabinoids tested (Fan et al., 1994).

Another variable currently associated with these subtle differences, as well as with any differences in the degree and time needed for a tolerance phenomenon to develop, is the different sensitivity of brain structures and functions involved in the specific effects studied, which produce those that responses exhibit a notable region-dependent pattern. An example can be found in the studies conducted by Spina et al. (1998). These authors reported that tolerance to the analgesic effect of WIN55,212-2 in mice developed after 5 days of daily exposure, while other effects, such as hypothermia and catalepsy, whose neuronal substrates are located in regions different than those involved in analgesic effects, needed 7 and 9 days, respectively, for the complete development of tolerance (Spina et al., 1998). Other examples of this region-dependency have been recently reviewed by Sim-Selley (2003). The reason for these region-dependent effects might be based on the observation that acute effects of cannabinoids reduced local cerebral glucose utilization with a pattern of responses that exhibited a clear dose-, time- and region-dependency (Freedland et al., 2002; Whitlow et al., 2002). Interestingly, the repetition of this treatment revealed the development of tolerance according to a region-dependent pattern (Whitlow et al., 2003). Another explanation, as will be detailed below, is that it is related to the region-dependent differences in the coupling of cannabinoid CB_1 receptors to GTP-binding proteins (Breivogel et al., 1997) since it has been suggested that receptor less efficiently coupled to their signaling mechanisms intensely develop desensitization while those efficiently coupled are more resistant to desensitization (Sim et al., 1996a,b; see Section 3.1).

Lastly, it is also important to remark that the tolerance phenomenon is not restricted to neurobiological effects of cannabinoids and that is also observed for some peripheral effects such as the case of the inhibition of splenocyte proliferation and natural killer cell activity, although this involves CB_2 rather than CB_1 receptors (Patrini et al., 1997). Other peripheral cannabinoid effects, such as hypotension and intestinal hypomotility, are also subjected to development of tolerance after prolonged treatments (see Pertwee, 2001; Sidney, 2002, for recent reviews).

3. Molecular mechanisms underlying cannabinoid tolerance

A long list of studies, carried out mainly during the past decade, have addressed the analysis of the molecular changes underlying the pharmacological tolerance developed after a prolonged exposure to plant-derived, synthetic or endogenous cannabinoids in adult individuals (for review, see Dewey, 1986; Pertwee, 1991, 1995; Maldonado, 2002). Thus, unlike earlier studies showing no changes in cannabinoid receptor binding or mRNA expression but that used conditions poorly favorable to reveal any changes in receptors (Westlake et al., 1991; Abood et al., 1993), our group (Rodríguez de Fonseca et al., 1994; Romero et al., 1995, 1997, 1998a,b, 1999; Corchero et al., 1999; Di Marzo et al., 2000; González et al., 2004) and others

(Oviedo et al., 1993; Rubino et al., 1994, 2000a,b; Sim et al., 1996a; Zhuang et al., 1998; Breivogel et al., 1999) have provided robust evidence that this pharmacological tolerance is mainly linked to changes in the availability of cannabinoid receptors, mainly the CB_1 receptor subtype, which is predominant in the CNS and involved in psychoactive properties of plant-derived cannabinoids. These changes in receptors would be more important, both qualitatively and quantitatively, than other phenomena also observed to explain cannabinoid tolerance such as increased metabolism of cannabinoid compounds (for review, see Pertwee, 1995) or adaptation of specific neuronal subpopulations (Hampson et al., 2003), which have been in general less studied and will not be addressed here with much detail.

3.1. Pharmacodynamic responses: changes in cannabinoid receptors and their endogenous ligands

As mentioned above, the elucidation of the mechanism(s) eliciting cannabinoid receptor regulation and adaptation of their endogenous ligands during chronic cannabinoid exposure is a phenomenon of particular interest for two reasons: (i) marijuana is widely used for its psychoactive properties, resulting in a chronic use by a percentage of the population, and (ii) regular use of cannabinoids as novel medicines is expected. There is great consensus that pharmacological tolerance for cannabinoids is caused by pharmacodynamic events (down-regulation/desensitization of cannabinoid receptors, mainly the CB_1 receptor subtype) (Oviedo et al., 1993; Rodríguez de Fonseca et al., 1994; Rubino et al., 1994, 2000a,b; Romero et al., 1995, 1997, 1998a,b, 1999; Sim et al., 1996a; Zhuang et al., 1998; Corchero et al., 1999; Di Marzo et al., 2000) rather than to enhanced metabolism (pharmacokinetic responses) or adaptation of specific neuronal subpopulations. These studies provided in vivo confirmation of data obtained in vitro using cultures of a neuroblastoma cell line (N18TG2) subjected to chronic exposure to cannabinoid agonists (Dill and Howlett, 1988). These authors found that the inhibition of cAMP accumulation in response to a chronic exposure to these drugs was diminished as compared with the response found after an acute exposure, suggesting the occurrence of desensitization of cannabinoid receptor-mediated inhibition of adenylyl cyclase (Dill and Howlett, 1988). Also exploring the impact of tolerance on cannabinoid receptor signaling, Shapira et al. (1998) conducted some in vitro studies that led to similar results. More recently, Rhee et al. (2000) found activation of some adenylyl cyclase isozymes after the chronic activation of CB_1 receptors in cells co-transfected with this receptor and individual adenylyl cyclase isozymes. Conversely, inhibitors of protein kinase A activity, were able to reverse the development of tolerance to cannabinoid-mediated antinociception and to other cannabinoid effects (Lee et al., 2003), suggesting that protein kinase A phosphorylates target proteins that are involved in the development and/or maintenance of cannabinoid tolerance, probably including CB_1 receptor itself (reviewed by Martin et al., 2004). Interestingly, inhibitors of other protein kinases were not effective with the only exception of src-family kinases (reviewed by Martin et al., 2004). As will be detailed below, these in vitro studies have had an adequate correlation in in vivo studies conducted by Galve-Roperh et al. (2002) and Rubino et al. (2004) who described that chronic cannabinoid exposure, at doses and times of treatment resulting in pharmacological tolerance, influences cannabinoid receptor signaling. These authors demonstrated a region-dependent modulation of ERK pathway, another signal transduction system coupled to CB_1 receptors (Galve-Roperh et al., 2002), by chronic Δ^9-THC administration (Rubino et al., 2004), that the authors proposed as a critical factor in triggering long-lasting neuronal adaptations following chronic cannabinoid exposure (see below). Rubino et al. (1997) also demonstrated that a chronic treatment with CP55,940 in rats reduced G-protein expression in many brain regions, although no changes were found in G-protein levels indicating that these changes do not appear to directly underly in receptor desensitization elicited by persistent receptor activation (Sim-Selley, 2003).

On the other hand, and in a similar way than for the variations described before for the phenomenon of pharmacological tolerance, there also exist variations in the onset, extent and duration of the pharmacodynamic responses caused by chronic cannabinoid administration, variations that can be attributed to the use of different types of cannabinoid agonists (with different affinities and/or pharmacological potencies), doses used and duration of treatment (see Pertwee, 1995; Sim-Selley, 2003; Martin et al., 2004, for review), lending support to the existence of potentially distinct mechanisms of receptor regulation (see Sim-Selley, 2003, for a recent review). An exception would be the endogenous ligand for cannabinoid receptors, anandamide, which due to its extremely poor stability (it is rapidly subjected to metabolic breakdown in arachidonic acid and ethanolamine) failed to elicit down-regulation/desensitization of cannabinoid receptors after prolonged treatment in several brain structures (Romero et al., 1995) or produced only partial effects (Rubino et al., 2000b) compared with the effects at multiple levels (receptor binding, receptor function and receptor synthesis) produced by classic and synthetic cannabinoids (Oviedo et al., 1993; Rodríguez de Fonseca et al., 1994; Rubino et al., 1994, 2000a; Romero et al., 1997, 1998a,b, 1999; Sim et al., 1996a; Zhuang et al., 1998; Corchero et al., 1999; Di Marzo et al., 2000). This is also supported by the fact that R-methanandamide, a more stable analog of anandamide, was however able to elicit down-regulation/desensitization to an extent almost comparable to classic and synthetic cannabinoids (see below and Romero et al., 1999).

Another interesting aspect is that the evidence described so far is compatible with the occurrence of a time-dependent and region-specific down-regulation/desensitization for CB_1 receptors under conditions of chronic cannabinoid administration (see Sim-Selley, 2003, for a recent review). The occurrence of region-dependent variations is indicative that CB_1 receptors, although structurally similar in all brain regions, would be regulated in a different manner in each region, or, in other words, that CB_1 receptors develop region-specific adaptations, which may have implications for the development of tolerance and dependence and indicate that CB_1 receptor adaptation involves multiple biochemical mechanisms (see Sim-Selley, 2003, for review). This region-dependent receptor plasticity, with areas showing high degree of changes and areas which did not respond, has been also found for other receptors activated by drugs of abuse, such as opioids (Law and Loh, 1999) and benzodiacepines (Klein and Harris, 1996). These regional differences would be, in most of the cases, small and related a priori to: (i) different tonic endocannabinoid activity, (ii) involvement of specific neuronal circuitries, (iii) formation of receptor dimers, (iv) variations in intracellular signaling mechanisms including different second messengers or differences in selectivity for certain Gα subunits, or (v) intervention of other non-CB_1 binding sites (see Sim-Selley, 2003). Zhuang et al. (1998) proposed the particular conditions of receptor regulatory mechanisms in each region as a plausible explanation for the considerable heterogeneity in the changes elicited by prolonged cannabinoid exposure in CB_1 receptors they found. These authors related these differences to the efficiency of effector systems coupled to CB_1 receptors (Zhuang et al., 1998), which they have already reported, that varies region-by-region (Breivogel et al., 1997). It appears reasonable that receptors better coupled to their signaling intracellular mechanisms are more resistant to develop adaptative changes than less-efficiently coupled receptors. This is the case of regions such as the hippocampus and the cerebellum, whose couplement is not highly effective but that respond more efficiently to chronic agonist treatment than hypothalamic and amygdaloid structures where receptor efficiency is maximal (Breivogel et al., 1997). This has been also observed for the case of μ-opioid receptors compared with cannabinoid CB_1 receptors (Sim et al., 1996a,b). Catalytic amplification is extremely higher for μ-opioid receptors than for CB_1 receptors, but the responses of this opioid receptor subtype to chronic agonist activation were anatomically discrete and relatively small in magnitude (Sim et al., 1996b) compared with the response in the case of CB_1 receptors (Sim et al., 1996a). Because of the importance of region-dependent differences in cannabinoid receptor regulation, below we will review all the studies published on this matter addressing the changes region-by-region, although we will address before some general ideas in relation with mechanisms involved in receptor regulation.

3.1.1. Cellular and molecular mechanisms involved in cannabinoid receptor regulation

The evidence reported so far support the notion that the primary effect caused by the prolonged occupancy of the cannabinoid receptor would be produced at the level of preexistent receptors rather than by affecting the receptor synthesis, which, if occurs, is always a secondary event. Several studies have extensively explored this issue in a series of time-course studies recording the effects of chronic Δ^9-THC exposure on CB_1 receptor binding and mRNA expression (Romero et al., 1998a; Zhuang et al., 1998) and WIN-55,212-2-stimulated [^{35}S]-GTPγS binding (Corchero et al., 1999; Breivogel et al., 1999). There is consensus that decreases in CB_1 receptor function (uncoupling or desensitization) precede those in receptor binding (down-regulation), and that both phenomena precede the changes in mRNA expression which only occurred in a few regions (Romero et al., 1998a; Zhuang et al., 1998; Breivogel et al., 1999). There is also consensus that the onset and the magnitude of these responses were always region-dependent (Romero et al., 1998a; Zhuang et al., 1998; Breivogel et al., 1999). The down-regulation/desensitization of CB_1 receptors would be elicited by a sequence of intracellular events triggered by the prolonged occupancy of the receptors and that we start now to understand. These are processes that develop very rapidly after the binding of the agonist to the receptor and affect receptor abundance and availability, and, consequently, the ability of agonists to generate an effective response. These would include: (i) covalent modification of receptors incorporated into the membrane, (ii) conformational changes, and (iii) internalization (recycling) of membrane fragments containing the CB_1 receptor–ligand complex, which, depending on the duration of agonist treatment, then might be sequestered within endosomes or degraded within lysosomes (Rinaldi-Carmona et al., 1998; Hsieh et al., 1999; Roche et al., 1999; Coutts et al., 2001). The intensity of these events would be, of course, influenced as a function of the relative potency of the different cannabinoid agonists used (Hsieh et al., 1999), and, in all cases, this leads to a progressive loss of the receptor ability to recognize their natural ligands and/or to activate signal transduction mechanisms, and, hence, originating pharmacological tolerance. The evidence accumulated so far indicate that desensitization and internalization of CB_1 receptors are mediated by phosphorylation of specific intracellular domains in the receptor that have been identified by several authors (García et al., 1998; Jin et al., 1999; Roche et al., 1999; Hsieh et al., 1999), whereas the loss of receptors may be due to increased degradation, although a decrease in synthesis, processing or expression of receptor function might be also involved. Receptor phosphorylation is catalyzed by a G-protein coupled receptor kinase (see Sim-Selley, 2003, for review), possibly the above mentioned kinases that have been involved in the development of cannabinoid tolerance. However, as Martin et al. (2004) recently reviewed, it is not clear whether these

protein kinases are directly involved in receptor regulation or whether these contribute to tolerance by modulating additional signaling pathways. Once phosphorylated, the receptor protein is then bound by β-arrestins, a group of multifunctional adaptor proteins involved in cellular mechanisms of regulation for G-protein coupled receptors (for review, see Gainetdinov et al., 2004). This prevents the receptor for interacting with and activating G-proteins leading to desensitization. β-Arrestins also direct the receptor to clathrin-coated pits followed by rapid endocytosis of receptor-containing vesicles in a dynamin-dependent fashion (Hsieh et al., 1999). By contrast, resensitization, which involves recycling of the receptor to the cell membrane, requires dephosphorylation after removal of agonist (Hsieh et al., 1999). These authors found that endosomal acidification is required for CB_1 receptor recycling and hypothesize that a conformational change in the receptor in acidified vesicles might permit receptor dephosphorylation and return to the cell membrane (Hsieh et al., 1999). In addition, recovery of cell surface receptors after prolonged agonist exposure would also require new protein synthesis, implying that receptors had been transported to lysosomes and degraded (Hsieh et al., 1999). Therefore, it can be concluded that short-term agonist exposure would produce reversible internalization, whereas long-term treatment would lead to irreversible internalization and receptor down-regulation (see Sim-Selley, 2003, for a recent review).

3.1.2. Regional differences in responses of cannabinoid receptors to chronic activation

As it has been largely discussed before, a repeated observation of studies addressing tolerance phenomena and cannabinoid receptor adaptations in the brain after chronic agonist exposure is the occurrence of region-dependent differences. For this reason, we have reviewed the CB_1 receptor responses to chronic activation region-by-region.

3.1.2.1. Basal ganglia. CB_1 receptors located in several neuronal subpopulations of the basal ganglia, such as striatal projection neuronal pathways and subthalamonigral neurons, play a role in the control of movement (Romero et al., 2002; Fernández-Ruiz et al., 2002). In these structures, Oviedo et al. (1993), by using autoradiographic techniques, and our group, by using binding of [^{3}H]-CP55,940 to brain membranes (Rodríguez de Fonseca et al., 1994) and further autoradiography (Romero et al., 1997, 1998a,b, 1999; Corchero et al., 1999), demonstrated that CB_1 receptor binding decreases after a chronic exposure to Δ^9-THC or other cannabinoid agonists in rats. These changes were paralleled by a pronounced reduction in the magnitude of motor inhibition caused by an acute cannabinoid treatment (Romero et al., 1997). Of particular interest is the report from Oviedo et al. (1993), who used regional Kd/Bmax analysis to clearly demonstrate down-regulation of cannabinoid receptors after chronic cannabinoid exposure. However, time-course studies have revealed that the onset of this reduction was slow needing at least three days of daily cannabinoid exposure in the case of receptors located in the lateral caudate-putamen and substantia nigra, and more than three days for receptors in the globus pallidus (Romero et al., 1998a). Reductions observed were only moderate (always lesser than 30% at the maximal time-course point) (Romero et al., 1998a), in particular in the case of the globus pallidus, entopeduncular nucleus and medial part of the caudate-putamen, which were exceedingly resistant (reductions lesser than 10%) to elicit down-regulation of cannabinoid receptors, even exhibiting no significant changes in some studies (Romero et al., 1997, 1998a, 1999). Similar results were published by Rubino et al. (2000a) in the two major striatal output nuclei, the globus pallidus and the substantia nigra, using chronic administration of CP55,940, but not using Δ^9-THC (Rubino et al., 2000c), and by Sim-Selley and Martin (2002) using WIN55,212-2.

Changes found in receptor density of Δ^9-THC-tolerant rats were accompanied in these structures by desensitization of cannabinoid-activated signal transduction mechanisms, measured by autoradiographic analysis of WIN-55,212-2-stimulated [^{35}S]-GTPγS binding (see Sim et al., 1996a). In addition, Rubino et al. (2000c) found an up-regulatory response of cAMP pathway (increased cAMP levels and protein kinase A activity), paralleled by reduced CB_1 receptor binding, in the striatum of Δ^9-THC-tolerant rats. However, in concordance with data of receptor density, the basal ganglia were among the regions where the decreases of WIN-55,212-2-stimulated [^{35}S]-GTPγS binding were more moderate (Sim et al., 1996a). For instance, using a short period of daily Δ^9-THC exposure, we reported that desensitization was evident in the substantia nigra but not in the globus pallidus (Romero et al., 1998b), however, Sim et al. (1996a), using a longer period of Δ^9-THC administration (21 days), found a small reduction of agonist-stimulated [^{35}S]-GTPγS binding in both structures. In time-course studies, we have observed that desensitization already occurs in the caudate-putamen from the first 24 h after the first cannabinoid administration reaching a maximal decrease after 14 days (Corchero et al., 1999), whereas Zhuang et al. (1998) reported a certain resistance in this structure and Breivogel et al. (1999) reported that desensitization occurred slowly in most of the basal ganglia, in particular in the globus pallidus where it did only occur after more than 14 days of daily Δ^9-THC exposure and it did not reach statistical significance (Breivogel et al., 1999). An additional aspect deserving comments is the fact that some studies have reported the occurrence of changes in receptor function (desensitization) in absence of detectable changes in the density (down-regulation), for instance, the studies conducted by Rubino et al. (2000b) who analyzed the changes of CB_1 receptors in the striatum and other brain regions of anandamide-tolerant rats. This apparent disagreement has been largely discussed in the studies by Childers and coworkers (Sim et al., 1996a) who stressed that down-

regulation (loss of binding) and desensitization (loss of receptor function) are separate processes, and that desensitization usually precedes down-regulation (see Section 3.1.1). However, a few studies have also reported losses of CB_1 receptor binding by chronic cannabinoid treatment with no changes in agonist-stimulated [^{35}S]-GTPγS binding (Romero et al., 1999), thus indicating the existence of alternative regulatory mechanisms for membrane receptors.

Lastly, Rubino et al. (1994) demonstrated that the reductions of CB_1 receptor binding and cannabinoid-activated signal transduction mechanisms in the basal ganglia were accompanied by reduction in mRNA levels for this receptor subtype in the caudate-putamen, measured by in situ hybridization in CP-55,940-tolerant rats. A time-dependent reduction in CB_1 receptor mRNA levels in the striatum after daily Δ^9-THC treatment was also reported by Zhuang et al. (1998), although we reported that changes in CB_1 receptor gene expression were always secondary (these usually appear after 7 days of chronic cannabinoid treatment) to changes in binding capacity for pre-existent receptors (Romero et al., 1998a; Corchero et al., 1999). By contrast, other studies have documented increases in CB_1 receptor mRNA levels in the caudate-putamen after a subchronic cannabinoid exposure (Romero et al., 1997) that were interpreted as a compensatory response directed to reduce the impact of the decrease in receptor density/efficiency, as suggested by Zhuang et al. (1998). It is possible that these up-regulatory responses occur only when short periods (<1 week) of repeated cannabinoid exposure were used (Romero et al., 1997) but not after long periods (>1 week) (Rubino et al., 1994). In addition, another important issue regarding the changes in CB_1 receptor mRNA levels is the fact that these reflect receptor synthesis in two different subpopulations of striatal neurons, including those reaching the *globus pallidus* (striatopallidal pathway) and those reaching the *substantia nigra/entopeduncular nucleus* (striatonigral pathway), that are regulated in a different way by several physiological or pathological influences (see Romero et al., 2002, for review). So, if the responses of these two neuronal subpopulations to chronic cannabinoid exposure are different, this might influence the changes recorded at the site where their cell bodies are located.

Most of the above studies were conducted with chronic treatments with Δ^9-THC, the prototypical cannabinoid present in *Cannabis sativa* derivatives. However, there are other cannabinoid agonists that were also able to elicit neuroadaptative changes of CB_1 receptors in the basal ganglia, although some small differences among these compounds and Δ^9-THC, likely due to their well-known pharmacodynamic or pharmacokinetic differences (Pertwee, 1997), were evident. This is the case of cannabinoid agonists such as AM356 (Romero et al., 1999), WIN55,212-2 (Sim-Selley and Martin, 2002), CP55,940 (Rubino et al., 2000a), and also anandamide (Rubino et al., 2000b). However, as mentioned above, in the case of this endogenous agonist, the changes did not affect CB_1 receptor binding (Romero et al., 1995; Rubino et al., 2000b), but did affect cannabinoid agonist-stimulated activation of GTP-binding protein (receptor function) although this did not result in changes in cAMP levels or in protein kinase A activity (Rubino et al., 2000b). This was paralleled by development of behavioral tolerance although to a lesser extent than other agonists likely because of its poor metabolic stability (Fride, 1995; Welch et al., 1995; Welch, 1997; Costa et al., 2000).

3.1.2.2. Hippocampus. CB_1 receptors located in hipocampal neuronal subpopulations are involved in the memory effects of cannabinoids (see Castellano et al., 2003, for review), and these effects develop tolerance likely due to the reductions in CB_1 receptor binding that take place in hippocampal structures (subfields of the Ammon's horn and dentate gyrus) after a chronic cannabinoid exposure (Romero et al., 1997, 1998a; Rubino et al., 2000c). In fact, the hippocampus was among those regions where the phenomenon of down-regulation of CB_1 receptors developed more rapidly, being necessary only 24 h after the first injection of Δ^9-THC to reach statistically-significant reductions (Romero et al., 1998a). The maximal effect was reached at 14 days (Romero et al., 1998a). In general, the different subfields (CA1, CA2 and CA3) of the Ammon's horn resulted to be more affected (losses of receptor binding were close to 40%) than the dentate gyrus where reductions were approximately 30% (Romero et al., 1997, 1998a). By contrast, the chronic administration of anandamide produced no changes (Rubino et al., 2000b) or an increase (Romero et al., 1995) in receptor binding in the hippocampus, but these differences might be attributable to the poor metabolic stability of this endocannabinoid agonist.

As in the case of the basal ganglia, Δ^9-THC-tolerant rats also showed a profound desensitization of cannabinoid-activated signal transduction mechanisms in hippocampal structures, measured by the autoradiographic analysis of WIN-55,212-2-stimulated [^{35}S]-GTPγS binding (Sim et al., 1996a; Sim-Selley, 2003). In this study, the different hippocampal structures were among the regions more markedly affected (Sim et al., 1996a) and, in these structures, the desensitization occurred very rapidly (already observable after 3 days of Δ^9-THC administration) (Zhuang et al., 1998; Breivogel et al., 1999), in concordance with receptor binding data (Romero et al., 1998a). Desensitization was also evident in hippocampal regions after chronic administration of other cannabinoid agonists such as CP55,940 (Rubino et al., 2000a), WIN55,212-2 (Sim-Selley and Martin, 2002) or anandamide (Rubino et al., 2000b).

From the above data, one may easily conclude that down-regulation/desensitization of CB_1 receptors in hippocampal structures underlies the tolerance for memory effects developed after repeated exposure to these compounds, despite some controversies on this effect mentioned above (Heyser et al., 1993; Deadwyler et al., 1995). However, in

contrast with the basal ganglia, the reductions in CB_1 receptor binding/function elicited by chronic activation of these receptors in hippocampal structures were not followed by reductions in mRNA levels (Rubino et al., 1994; Romero et al., 1995, 1997, 1998a). There were only two exceptions to this observation. One was the study by Zhuang et al. (1998) who described a biphasic pattern, an early reduction followed by a late increase, for CB_1 receptor mRNA levels measured by RT-PCR in the hippocampus after long-term exposure to Δ^9-THC. In addition, we observed that chronic administration with AM356 in rats reduced CB_1 receptor mRNA levels in the Ammon's horn but did not produce any changes in CB_1 receptor binding and activation of GTP-binding proteins (Romero et al., 1999), being this study the only one reporting changes in receptor synthesis with no changes in receptor binding and function.

3.1.2.3. Cortical regions. Reductions in CB_1 receptor binding and WIN-55,212-2-stimulated [^{35}S]-GTPγS binding did also occur in cortical structures of Δ^9-THC-tolerant rats (Sim et al., 1996a; Romero et al., 1998a; Rubino et al., 2000c). This occurred in both the superficial (I–II) and the deep (V–VI) layers but only this latter structure showed a rapid response (reductions appeared at 24 h after first injection) and of great magnitude (reductions were approximately 30% after 14 days of daily exposure) (Romero et al., 1998a). As a result of this down-regulation/desensitization phenomenon, there was an enhancement of cAMP pathway (increased cAMP levels and protein kinase A activity) in the cerebral cortex of Δ^9-THC-tolerant rats (Rubino et al., 2000c). Chronic administration of AM356 was not effective to reduce CB_1 receptor binding and WIN-55,212-2-stimulated [^{35}S]-GTPγS binding in cortical structures (Romero et al., 1999) whereas WIN55,212-2 produced a marked reduction in these two parameters in mice (Sim-Selley and Martin, 2002). In addition, CP55,940 produced a marked reduction of CB_1 receptor binding (Rubino et al., 2000a) and anandamide reduced cannabinoid agonist-stimulated activation of GTP-binding protein (Rubino et al., 2000b). However, as described for the hippocampus and opposite to the basal ganglia, the effects of all these agonists were not accompanied by reductions in mRNA levels (Rubino et al., 1994; Romero et al., 1998a,b, 1999).

3.1.2.4. Cerebellum. Cannabinoid-induced motor effects also include ataxia and incoordination, which are likely mediated by the activation of CB_1 receptors located in the cerebellum (Patel and Hillard, 2001; De Santy and Dar, 2001). These effects also develop tolerance after a prolonged treatment (Martin et al., 1975) and this tolerance is possibly caused by the significant reductions in CB_1 receptor binding and/or WIN-55,212-2-stimulated [^{35}S]-GTPγS binding measured in the cerebellum of Δ^9-THC-tolerant rats (Sim et al., 1996a; Romero et al., 1997, 1998b). Down-regulation/desensitization occurred more slowly (starting more than 3 days after the onset of Δ^9-THC treatment) than in the case of the hippocampus, a region where desensitization is rapid, although it reached a similar extent (Breivogel et al., 1999). Some authors have interpreted this fact on the basis that the cerebellum would contain a reserve for CB_1 receptor allowing a reduced loss of cannabinoid binding sites (see Sim-Selley, 2003, for review). Down-regulation/desensitization of CB_1 receptors in the cerebellum were also shown after chronic treatments with CP55,940 (Rubino et al., 2000a), WIN55,212-2 (Sim-Selley and Martin, 2002) or AM356 (Romero et al., 1999) using autoradiographic analyses in rats. In addition, Rubino et al. (2000b) reported that a chronic treatment with the endogenous agonist anandamide did not affect CB_1 receptor binding in the cerebellum but did affect receptor function, although, as also found in the basal ganglia, this did not result in changes in cAMP levels or in protein kinase A. This contrasts with the data reported by the same group in Δ^9-THC-tolerant rats where a significant reduction in CB_1 receptor binding in the cerebellum was accompanied by upregulation of the cAMP pathway (increased cAMP levels and protein kinase activity) (Rubino et al., 2000c), thus stressing the pharmacokinetic differences between Δ^9-THC and anandamide. This also contrasts with a previous study by Fan et al. (1996), who, using membrane binding techniques, reported a strong reduction of CB_1 receptor binding in mice chronically treated with CP55,940 (Fan et al., 1996). However, these authors also recorded the inhibitory effect of this cannabinoid on adenylyl-cyclase activity and found that the down-regulation was not accompanied by a decrease in receptor function (Fan et al., 1996). The authors concluded that, as this assay was conducted in vitro, it would be possible that it did not correctly reflect the cannabinoid receptor/adenylyl cyclase coupling in the tolerant state (Fan et al., 1996). Again, the changes in receptors found in the cerebellum of cannabinoid-tolerant individuals were not accompanied by reductions in mRNA levels (Rubino et al., 1994; Romero et al., 1997, 1998b, 1999). However, this also contrasts with the above mentioned study by Fan et al. (1996), who did observe an increase in mRNA levels, measured by Northern blot, in the cerebellum of mice chronically exposed to CP55,940 that the authors interpreted as a compensatory response for receptor loss. Partly in concordance with this last observation, Zhuang et al. (1998) reported a biphasic pattern, early decrease followed by a late increase, for CB_1 receptor mRNA levels in the cerebellum of Δ^9-THC-treated rats. Variations in animal species used, type, dose and duration of cannabinoid treatment chosen and other variables should account for these differences.

3.1.2.5. Limbic regions. The different limbic structures, including nucleus accumbens, septum nuclei, amygdala and others, contain moderate levels of CB_1 receptors, so that these are involved in the control of emotions, and in the

response to rewarding and motivational stimuli. These also experienced reductions in CB_1 receptor binding and WIN-55,212-2-stimulated [^{35}S]-GTPγS binding after a chronic cannabinoid exposure (Rodríguez de Fonseca et al., 1994; Sim et al., 1996a; Romero et al., 1998a; Sim-Selley and Martin, 2002). However, these regions were among the structures that showed more resistance to this phenomenon in some studies, in particular the basolateral part of the amygdala and, to a lesser extent, the nucleus accumbens (Romero et al., 1998a). This resistance appears in that the reductions: (i) were usually very modest (Rodríguez de Fonseca et al., 1994; Romero et al., 1998a) although not in all studies (Romero et al., 1997) — the dose and the type of cannabinoid agonist used may be more critical factors here than in other regions —, (ii) needed that the treatment prolongs for various days (at least 3 days or more; see Romero et al., 1998a), and (iii) were not evident in some studies (Romero et al., 1995, 1999; Di Marzo et al., 2000). It is important to note that limbic structures are the only region where the levels of endocannabinoids increased significantly in cannabinoid-tolerant rats (Di Marzo et al., 2000; see details in Section 3.1.3), which might be indicative of the occurrence of a different regulation of endocannabinoids and their receptors in response to a chronic activation in these regions compared with other areas. In addition, the reduction in CB_1 receptor binding and activation of GTP-binding proteins in these regions were not followed in any case by reductions in mRNA levels (Romero et al., 1998a, 1999).

3.1.2.6. Hypothalamic structures. CB_1 receptors are slightly to moderately abundant in different hypothalamic structures (Fernández-Ruiz et al., 1997), although these appear to be actively coupled to their signaling mechanisms, as demonstrated by Breivogel et al. (1997). These receptors mediate the effects of cannabinoids by reducing the release of different anterior pituitary hormones (prolactin, gonadotrophin, growth hormone) and increasing corticotropin secretion (for review, see Wenger and Moldrich, 2002). CB_1 receptors located in the preoptic area have been also involved in the hypothermic effects of cannabinoid agonists (Sim-Selley, 2003). Some studies reported that a prolonged treatment develops tolerance to neuroendocrine effects of Δ^9-THC (Smith et al., 1983; Rodríguez de Fonseca et al., 1991; see Murphy et al., 1998; Brown and Dobs, 2002, for review). However, tolerance was not evident with other cannabinoid agonists. This is the case of the reduction in prolactin and LH secretion caused by an acute dose of AM356 (De Miguel et al., 1998), which did not develop tolerance when the treatment was repeated for 5 days, although it is possible that this emerges after a longer period of treatment. This was paralleled by no changes in CB_1 receptor binding and mRNA levels in several hypothalamic regions (ventromedial hypothalamic nucleus, arcuate nucleus, medial preoptic area) after acute or chronic treatment with AM356 (De Miguel et al., 1998) or Δ^9-THC (Romero et al., 1995). The same absence of changes in CB_1 receptor binding and mRNA levels was observed in the ventromedial hypothalamic nucleus during a period of 1 up to 14 days of daily Δ^9-THC administration (Romero et al., 1998a), although 5 days of Δ^9-THC exposure did elicit a marked down-regulation (more than 50% of decrease) of CB_1 receptors in the arcuate nucleus (Romero et al., 1997). By contrast, 18 days of daily administration of CP55,940 in rats did reduce CB_1 receptor mRNA levels in the ventromedial hypothalamic nucleus and CB_1 receptor binding in this structure and also in the arcuate nucleus (González et al., 1999), whereas chronic treatment with WIN55,212-2 in mice markedly reduced CB_1 receptor binding and activation of GTP-binding proteins in the whole hypothalamus (Sim-Selley and Martin, 2002). This indicates that the duration of the chronic treatment or the type of agonists employed might be also key variables in this region.

Further demonstration that CB_1 receptors are present in the pituitary gland (González et al., 1999; Wenger et al., 1999), allowing a direct control by cannabinoids of the hormone secretion by this gland, prompted to look also how these receptors respond to prolonged activation. We found that 18 days of daily administration of CP55,940 did increase CB_1 receptor mRNA levels in the anterior pituitary but not in the intermediate lobe (González et al., 1999). However, the time-course of this effect was probably biphasic since a further experiment with Δ^9-THC revealed a reduction of mRNA levels at early times (1–3 days after the onset of treatment) followed by normalization around 7 days, and an up-regulatory response after 2 weeks (González et al., 1999).

3.1.2.7. Miscellaneous. Reductions in CB_1 receptor binding and/or activation of GTP-binding proteins after chronic cannabinoid administration did also occur in other brain regions, such as several thalamic structures (Sim-Selley and Martin, 2002) and the central gray substance (Romero et al., 1997; Sim-Selley and Martin, 2002). This last region has been involved in the supraspinal nociceptive control and, hence, it is related to the tolerance observed for the analgesic effects of cannabinoids (Rubino et al., 2000c). It is not expected that these changes in receptor binding are associated with reductions in mRNA levels, as observed in other regions (Rubino et al., 1994; Romero et al., 1998a). In addition, Sim et al. (1996a) reported that desensitization of cannabinoid-activated signal transduction mechanisms in the central gray substance of Δ^9-THC-tolerant rats was not significant. To our knowledge, little information is available regarding the effects of chronic cannabinoid treatments on CB_1 receptors at other brain regions involved in supraspinal nociception and, in particular, involved in spinal nociception (see Sim-Selley, 2003, for review), despite the importance of these data considering the expected regular use of cannabinoids in patients with chronic pain.

Tolerance to peripheral effects of cannabinoids, such as the immunosuppressive effects of CP55,940 (Patrini et al., 1997), is also pharmacodynamic in nature, so it is associated with a loss of functional cannabinoid receptors in rat spleen during chronic treatment, as observed by Massi et al. (1997), leading to diminished biological responses. However, in this case, CB_2 rather than CB_1 would be the receptor subtype presumably involved in tolerance for the immunosuppresive effects of cannabinoids.

3.1.3. Changes in endocannabinoid ligands

In addition to changes in receptor binding sites, it is likely to expect that tolerance to cannabinoids is also associated with adaptative changes in the synthesis, release and/or metabolism of the two major endogenous ligands for these cannabinoid receptors, anandamide and 2-arachidonoyl-glycerol. However, this question has been explored only very recently using rats rendered Δ^9-THC-tolerant through a paradigm of daily injections of Δ^9-THC (10 mg/kg) during 7 days (Di Marzo et al., 2000; González et al., 2004). Our data indicated that Δ^9-THC-tolerant rats exhibited changes in the contents of these two endocannabinoids but these were restricted only to a few regions (Di Marzo et al., 2000; González et al., 2004). We found that 2-arachidonoyl-glycerol contents markedly decreased in the striatum of Δ^9-THC-tolerant rats but no changes were found in other brain regions, such as the cerebral cortex, limbic forebrain, hippocampus, diencephalic structures, midbrain, cerebellum, and brainstem (Di Marzo et al., 2000; González et al., 2004). Anandamide levels were also reduced in the striatum but the levels of its metabolic precursor, N-arachidonoyl-phosphatidylethanolamine, did not change (Di Marzo et al., 2000). The same lack of changes was observed for anandamide levels in the remaining brain regions (Di Marzo et al., 2000; González et al., 2004), with the only exception of the limbic forebrain where the levels of anandamide increased dramatically accompanied by a non-significant trend toward an increase in the levels of its metabolic precursor (Di Marzo et al., 2000). Interestingly, no changes in CB_1 receptor binding and cannabinoid-activated GTP-binding proteins were recorded in this brain region of Δ^9-THC-tolerant rats used in that study, whereas reductions were evident in those regions where endocannabinoid levels were not altered (Di Marzo et al., 2000). This was another example of region-dependent changes in endocannabinoid signaling in Δ^9-THC-tolerant animals, that, assuming the occurrence of these changes only in motor and limbic regions, might be indicative of the important role played by these endogenous ligands and their receptors in specific brain processes, such as the control of movement (Fernández-Ruiz et al., 2002) and, in particular, the phenomenon of drug addiction (Gardner and Vorel, 1998; Manzanares et al., 1999; Hungund and Basavarajappa, 2000), which involves specific nuclei located in limbic structures.

3.2. Metabolism of cannabinoids during chronic treatments

There is some evidence that chronic administration of cannabinoids alters drug absorption, bioavailability, tissue distribution, metabolism or excretion of cannabinoids in the brain and the periphery (reviewed by Pertwee, 1991) giving a priori support to the idea that cannabinoid tolerance might be pharmacokinetic. However, as largely demonstrated above, most of the experimental evidence indicate that tolerance is pharmacodynamic in nature rather than pharmacokinetic. This is concordant with some early studies by Martin and coworkers who found no relevant differences, or these were too small, in cellular and subcellular distribution of Δ^9-THC in the brain and peripheral tissues, as well as in plasma concentrations for this cannabinoid, between tolerant and non-tolerant dogs (Martin et al., 1976). Similar conclusions were done in human studies (Hunt and Jones, 1980). Some more recent studies (Rodríguez de Fonseca et al., 1991; Bornheim et al., 1994; Costa et al., 1996; for review, see Pertwee, 1995), however, have provided evidence of a reduced bioavailability of active cannabinoids because of increased metabolism after chronic treatments. This would not be an exclusive event underlying tolerance but most authors accept that would add to the pharmacodynamic events described in the above section. Thus, Rodríguez de Fonseca et al. (1991) described that plasma levels of Δ^9-THC were significantly reduced after 7 and, in particular, 15 days of a daily exposure to this cannabinoid compared with the levels measured after a single administration. This suggests that Δ^9-THC would be more rapidly and efficiently metabolized, possibly by classic hepatic enzymes involved in cannabinoid metabolism, after a prolonged period of exposure to this cannabinoid. In support of this option, another plant-derived cannabinoid, cannabidiol, has been reported to induce some isoforms of cytochrome P450, an enzymatic complex involved in the metabolism of different types of xenobiotics, including cannabinoids, in the liver and also in other tissues (Bornheim et al., 1994). Finally, Costa et al. (1996) described a reduced bioavailability of a synthetic cannabinoid agonist, CP55,940, after a chronic treatment with this compound in rats. They found that CP55,940 also increased the activity of microsomal cytochrome P450 oxidative system (Costa et al., 1996).

3.3. Neuronal adaptation after chronic cannabinoid exposure

As mentioned above, a few studies have also suggested the occurrence of phenomena of adaptation of specific neuronal subpopulations after chronic cannabinoid exposure or of other type of phenomena different than classic pharmacodynamic or pharmacokinetic tolerance (Spina et al., 1998; Wu and French, 2000; Hampson et al., 2003; Corchero et al., 2004). This would be an indirect mechanism for cannabinoid tolerance produced beyond the primary site

of action of cannabinoids (i.e., cannabinoid receptor), acting, for example, by altering transmission along some neuronal pathways mediating some specific effects of cannabinoids (reviewed by Pertwee, 1991). One option is the case of nitric oxide which has been proposed to play a role in the development of tolerance for the hypothermic and cataleptic effects of cannabinoids (Spina et al., 1998). This proposal is based on the fact that inhibition of nitric oxide synthase abolished the development of tolerance for both effects but not for the analgesic effects of cannabinoids (Spina et al., 1998). Therefore, an increased formation of nitric oxide in the cerebral areas involved in the hypothermic and cataleptic effects of cannabinoids should be concomitant with the development of tolerance for both effects (Spina et al., 1998).

Other relevant case concerns the memory effects of cannabinoids. Thus, WIN55,212-2 (Hampson et al., 2003) and Δ^9-THC (Deadwyler et al., 1995) developed tolerance to their memory disruptive effects, although several weeks were needed. The authors related this tolerance phenomenon to adaptation (altered firing) of a select population of hippocampal neurons (i.e., entorhinal cortical projections to CA1 and CA3 subfields in the Ammon's horn) involving a CB_1 receptor-mediated decrease in GABA release via a retrograde action which would be sensitive to chronic cannabinoid exposure (Hampson et al., 2003). However, studies by Gessa and coworkers did not show tolerance to the inhibitory effects of Δ^9-THC on acetylcholine release in the hippocampus (Carta et al., 1998; Nava et al., 2001), which has been reported to play an important role in memory impairment caused by cannabinoids (Mishima et al., 2002). Gessa and coworkers (Carta et al., 1998; Nava et al., 2001) related their observations to the early studies reporting lack of tolerance to the memory impairment caused by Δ^9-THC in monkeys (Ferraro and Grilly, 1973), as well as to clinical observations in humans that memory impairment does not disappear or reduce in chronic marijuana users (Chait and Perry, 1994; Heishman et al., 1997). However, other studies reported tolerance to the amnesiac effects of cannabinoids (see Sim-Selley, 2003, for a recent review), stating that differences in dosage and duration of chronic treatment may account for these discrepancies.

As noted for memory effects of cannabinoids, neuronal adaptation processes in response to tolerance phenomena depend on the specific subpopulations involved. Another example of this can be found in the case of specific subpopulations of mesencephalic dopaminergic neurons. Thus, Wu and French (2000) showed that, during chronic treatment with Δ^9-THC, the neurons of the substantia nigra pars compacta developed tolerance to the effects of this cannabinoid, while the neurons of ventral-tegmental area did not, exhibiting continued increases of firing in rats when challenged repeatedly with Δ^9-THC. The reason for these differences is still unknown, since the mechanisms by which chronic Δ^9-THC treatment does not induce tolerance in ventral-tegmental neurons remains unknown, but it (these) may account for the lack of tolerance to the euphorigenic effects induced by marijuana (Dewey, 1986; Pérez-Reyes et al., 1991).

A last relevant case, concerning the neuronal adaptative processes elicited by chronic cannabinoid exposure, may be related to the involvement of opioidergic neurons in some effects of cannabinoids. Cannabinoids and opioids share many pharmacological effects and there are many examples of cross-tolerance or synergic effects between both types of substances, in particular for their antinociceptive effects (see Manzanares et al., 1999; Maldonado and Rodríguez de Fonseca, 2002, for review). Several studies have demonstrated that chronic administration of different cannabinoids upregulate the opioid system in different brain areas related to motor function, cognitive and emotional responses, and neuroendocrine control (see Manzanares et al., 1999; Corchero et al., 2004, for recent reviews), and this might generate neuronal adaptative phenomena. By contrast, in vitro studies have revealed examples of cross-desensitization and down-regulation between opioid and cannabinoid agonists after prolonged exposures although the symmetry of the response was cell type-dependent (Shapira et al., 1998, 2003).

4. Do cannabinoids produce physical dependence and/or abstinence?

Tolerance and dependence often develop concomitantly and, in some cases, the severity of the physical dependence/withdrawal syndrome is directly related to the magnitude of tolerance. Therefore, assuming that chronic cannabis use leads to adaptative changes in endocannabinoid signaling, it may also be assumed that these changes contribute to the development of cannabis physical dependence (Piomelli, 2004). However, while there is a lot of studies which strongly suggest that cannabinoid tolerance is consequence of region-dependent losses in cannabinoid CB_1 receptor binding, mRNA expression and agonist-stimulated G protein activity, and also of adaptative changes of endocannabinoid contents (see above), less data exist on the response of these adaptative changes when cannabinoid administration is abruptly terminated after a prolonged period of daily exposure (see Maldonado, 2002; Lichtman and Martin, 2002, for recent reviews). This is a relevant issue for cannabinoid consumption in humans, since there is no general agreement about whether cannabinoid tolerance has elements of physical dependence (for review, see Kalant, 2004), as it has been demonstrated for other drugs of abuse such as morphine (for review, see Williams et al., 2001), alcohol (for review, see Weiss and Porrino, 2002) or cocaine (for review, see Dackis and O'Brien, 2001). For these drugs, the interruption of chronic administration results in the spontaneous occurrence of somatic and/or neurovegetative signs of abstinence in laboratory animals.

However, this does not appear to be the case for cannabinoids, presumably because of their particular pharmacokinetic properties (the same that influence cannabinoid self-administration by laboratory animals; Justinova et al., 2005): (i) late onset, (ii) greater duration, and (iii) slow metabolic clearance (for review, see Pertwee, 1991, 1995, 1997).

4.1. Withdrawal signs in cannabinoid-tolerant animals

As it frequently happens with drugs that have a long duration of action, most of the studies with cannabinoids failed to elicit spontaneously any relevant somatic and/or neurovegetative signs of abstinence in laboratory animals (for review, see Maldonado, 2002; Lichtman and Martin, 2002). However, some withdrawal signs have been reported to occur in humans (Jones et al., 1981) and non-human primates (Beardsley et al., 1986), even though these are mild compared to those observed, for example, with opioids (for review, see Hollister, 1986; Pertwee, 1991; Kalant, 2004), and these do not appear in all individuals (Perkonigg et al., 1999). Several reports (Solowij et al., 2002; Budney et al., 2004; Haney et al., 2004; Kalant, 2004) reviewed recently this issue in humans and concluded that withdrawal signs reliably follow discontinuation of chronic heavy use of cannabis. For these authors, common symptoms for these "heavy" marijuana users are primarily emotional and behavioral (i.e., irritability, anxiety, attentional deficits, and sleep difficulties), although anorexia, weight loss, stomach and muscle pain, nausea and physical discomfort are also frequently reported (Solowij et al., 2002; Budney et al., 2004; Haney et al., 2004; Kalant, 2004). The situation in animal studies remains, however, unconclusive (Lichtman et al., 2002, for review). Only two recent studies (Aceto et al., 2001; Oliva et al., 2003) reported the occurrence of spontaneous cannabinoid abstinence in rodents but both studies, instead herbal cannabinoids, used synthetic agonists of higher pharmacological potency (Oliva et al., 2003) or with significantly different pharmacokinetics (Aceto et al., 2001), thus waning the relevance of these observations as regards to the situation in humans. However, an effective means of demonstrating physical dependence in the absence of spontaneous abstinence is to abruptly terminate the agonist action, and to elicit abstinence, by challenging agonist-tolerant animals with a receptor antagonist. Thus, a large number of studies have demonstrated that the blockade of CB_1 receptors with rimonabant (SR141716), a selective antagonist for this receptor subtype, elicits a series of withdrawal signs in cannabinoid-tolerant animals (Aceto et al., 1995, 1996; Tsou et al., 1995; Hutcheson et al., 1998; Cook et al., 1998; Rubino et al., 1998; Anggadiredja et al., 2003), signs that were somatic, with no relevant neurovegetative changes (Maldonado, 2002; Lichtman and Martin, 2002). Indeed, data obtained in CB_1 receptor knockout mice showed that rimonabant administration after chronic Δ^9-THC administration failed to precipitate any withdrawal signs, thus indicating that somatic signs of abstinence are CB_1 receptor-mediated (Ledent et al., 1999). The most characteristic somatic signs were motor in nature and included wet dog shakes, head shakes, facial rubbing, front paw tremor, ataxia, hunched posture, body tremor, ptosis, piloerection, hypolocomotion, mastication, licking, rubbing, and scratching (reviewed by Maldonado and Rodríguez de Fonseca, 2002). This is concordant with a recent in vivo study by Pillay et al. (2004) who reported changes in motor cortical areas, in particular, attentional areas related to motor function, in response to a motor task in abstinent, chronic, cannabis smokers.

However, data collected after precipitation with rimonabant of a withdrawal syndrome in cannabinoid-tolerant rodents have been questioned in two aspects. First, they used potent synthetic cannabinoid agonists, with a significantly greater pharmacological potency or different pharmacokinetic properties compared to plant-derived cannabinoids (Rodríguez de Fonseca et al., 1997; Rubino et al., 1998, 2000a). Some studies used Δ^9-THC but, then, the dose and/or the timing used strongly exceeded the pattern of cannabis use in humans (reviewed recently by Maldonado, 2002). A second important issue is that not all studies controlled the effects of rimonabant, which has been reported to also act as an inverse agonist (Pertwee, 1997; Rubino et al., 2000d), in non-tolerant rats (Diana et al., 1998; Cook et al., 1998), which complicated the correct interpretation of these data. In a recent study addressed to avoid the above two problems (González et al., 2004), we reported the occurrence of overt behavioral signs, accompanied by a sort of endocrine and molecular changes that will be discussed below, elicited by SR141716 in rats rendered Δ^9-THC-tolerant by a chronic treatment with this cannabinoid at moderate doses. These withdrawal signs would possibly reflect the occurrence of a pharmacologically-induced withdrawal syndrome since these were not so evident in non-tolerant rats (González et al., 2004). This is the case of some responses found in the open-field test that reproduced the results found by other authors showing that the withdrawal syndrome elicited by rimonabant develops according to a pattern of motor alterations with no appearance of neurovegetative signs (Aceto et al., 1995, 1996; Tsou et al., 1995; Hutcheson et al., 1998; Cook et al., 1998; Rubino et al., 1998). Thus, the challenge with rimonabant in Δ^9-THC-tolerant rats produced increased spontaneous ambulation and occurrence of non-ambulatory activities, such as tremor, turning, retropulsion and stereotypes, and reduced inactivity. Tremor and, particularly, retropulsion were two of the behavioral signs which appeared more enhanced in Δ^9-THC-tolerant rats challenged with SR141716 (González et al., 2004), in concordance with previous studies (Tsou et al., 1995; Aceto et al., 1996). By contrast, the occurrence of scratching was attenuated in Δ^9-THC-tolerant rats challenged with SR141716 compared with the response found in non-tolerant animals, as also seen in mice (Cook et al., 1998).

It is important to mention that the cannabinoid withdrawal effects reported in humans and laboratory animals (see above) are transient and can be suppressed or attenuated by re-administration of cannabis or Δ^9-THC (for review, see Pertwee, 1991). On the other hand, the recent discovery and characterization of the endocannabinoid signaling system allows to explain, as described above, the molecular bases of pharmacological properties of cannabis and cannabinoids (Lichtman and Martin, 2002; Piomelli, 2004). Both facts lend support to the development of therapeutic strategies based on the pharmacological management of this system to alleviate marijuana dependence, although the evidence so far remains unconclusive (see Piomelli, 2004). In this sense, a recent report by Haney et al. (2004), showing that marinol (oral Δ^9-THC) may be able to work in a replacement therapy, has provided significant promise, although this option is limited due to the occurrence of undesirable psychotropic and cardiovascular side effects. A way to avoid these problems might be the use of the recent developed agents capable to protect endocannabinoids (transport inhibitors, FAAH inhibitors) from endogenous inactivation (see Piomelli, 2004, for review). However, there are no published evidence on this possibility neither in laboratory animals nor in humans, so this issue will be a major challenge for the future. If this strategy works, it will add to other strategies presently examined for the treatment of cannabis dependence, such as the case of lithium. The administration of lithium blocked all withdrawal signs elicited by CB_1 receptor blockade in rats chronically treated with HU-210, possibly through the activation of oxytocinergic neurons within the CNS (Cui et al., 2001). This effect of lithium does not seem to be related to its activity as mood stabilizer since another mood stabilizers, such as sodium valproate, were not effective (Cui et al., 2001).

4.2. Molecular mechanisms underlying cannabinoid dependence/abstinence

The above somatic signs reported in Δ^9-THC-tolerant animals challenged with rimonabant were accompanied by a series of molecular events, that are characteristic of the abstinence to other drugs (for review, see Koob, 1999; Georges et al., 2000; Sarnyai et al., 2001), such as: (i) as c-*fos* induction (Rodríguez de Fonseca et al., 1997); (ii) increased CRF release in the central nucleus of the amygdala (Rodríguez de Fonseca et al., 1997); (iii) changes in adenylate cyclase/cAMP signaling system in the cerebellum (Hutcheson et al., 1998; Tzavara et al., 2000; Rubino et al., 2000b), a fact also found in vitro (Rhee et al., 2000); and (iv) decreased dopamine release in the nucleus accumbens (Diana et al., 1998; Tanda et al., 1999), which may be related to the aversive and dysphoric consequences of cannabinoid withdrawal (Maldonado and Rodríguez de Fonseca, 2002).

We have also examined possible changes in plasma prolactin and corticosterone levels, which are stress hormones affected during drug withdrawal (Pickworth and Fant, 1998; Zorrilla et al., 2001), and we found that the administration of SR141716 increased plasma levels of both prolactin and corticosterone in non-tolerant rats, whereas Δ^9-THC-tolerant rats did not respond in the case of prolactin and exhibited a trend toward an increase in the case of corticosterone, with high individual variations which prevented the effect from reaching statistical significance (González et al., 2004). We also examined some molecular parameters, also controlled during the abstinence to opioids, alcohol or psychostimulants (for review, see Koob, 1999; Georges et al., 2000; Sarnyai et al., 2001), such as the gene expression of proenkephalin (a marker of opioid activation), c-*fos* (a marker of neuronal activation) and CRF (a marker of stress) in specific brain regions. The first two parameters were slightly affected by induction with rimonabant of a pharmacological withdrawal syndrome in Δ^9-THC-tolerant rats (González et al., 2004). However, the response found for CRF-mRNA levels in the paraventricular hypothalamic nucleus, the structure where cell bodies of CRF-containing neurons in the brain are located, appeared very relevant. CRF gene expression increased after challenging with SR141716 in the case of Δ^9-THC-tolerant rats, but the response in the case of non-tolerant rats was exactly the opposite (González et al., 2004). This observation is consistent with the data reported previously by Rodríguez de Fonseca et al. (1997), who demonstrated an increased CRF release from the medial amygdaloid nucleus in SR141716-challenged cannabinoid-tolerant rats. This increase has been also reported to occur during withdrawal from other drugs of abuse and has been related to the activity of neuronal substrates underlying stress and anxiety responses typical of drug abstinence (for review, see Sarnyai, 1998; Koob, 1999).

We have also assessed the changes in the levels of endocannabinoids in various brain regions which, to this date, had not been analyzed during cannabinoid abstinence (González et al., 2004), although some studies had already measured the changes in CB_1 receptor binding and cannabinoid-activated GTP-binding proteins in this situation (Rubino et al., 1998, 2000a; Breivogel et al., 2003). For instance, Rubino et al. (2000a) found that a time longer than 24 h after the rimonabant challenge was needed for the complete recovery of adaptative changes produced during tolerance in CB_1 receptors (Rubino et al., 2000c). By contrast, Breivogel et al. (2003) found that CB_1 receptor binding and cannabinoid-activated GTP-binding proteins remained decreased in the cerebral cortex, hippocampus, basal ganglia and cerebellum of rats subjected to chronic Δ^9-THC treatment and further discontinuation of this treatment for 25 h. However, it is possible that a "wash out" period of only 25 h was not enough to assess a complete removal of Δ^9-THC from the brain due to lipophylic characteristics of cannabinoids. This is supported by the fact that CB_1

receptor blockade with rimonabant at this time still produced withdrawal responses of similar magnitude (Breivogel et al., 2003) than when the antagonist was administered before the onset of discontinuation phase (Aceto et al., 1995; Breivogel et al., 2003). As regards to endocannabinoid ligands, the most interesting observation was that most of the changes elicited by cannabinoid tolerance (Di Marzo et al., 2000; González et al., 2004), in concordance with the above-described data reported by Rubino et al. (1998, 2000a) on CB_1 receptors, were reversed by administration of rimonabant, with the exception of anandamide in the midbrain and 2-arachidonoyl-glycerol in the hippocampus (González et al., 2004). By contrast, rimonabant had no effect in non-tolerant rats (González et al., 2004). These region-dependent differences in the response of Δ^9-THC-tolerant rats to SR141716 might be related to the selectivity of behavioral signs exhibited by cannabinoid abstinent animals, which were mainly of motor nature (González et al., 2004). The same region-dependent differences in the response elicited by an acute administration of rimonabant to cannabinoid-tolerant rats were described by Rubino et al. (2000a) who looked at the CB_1 receptor binding, with regions, such as the cerebral cortex and the caudate-putamen, exhibiting complete recovery to values similar than controls, and other regions, such as the cerebellum and the hippocampus, which still remained decreased.

5. Are cannabinoids a gateway drug? Evidence from laboratory animals

Another controversial aspect regarding cannabinoid addiction is the proposed enhanced vulnerability to consume other drugs of greater addictive power, such as morphine or cocaine, that cannabinoid tolerance may generate, and that allowed some authors to develop the theory of "cannabis as a gateway drug" (Kandel et al., 1997; Fergusson and Horwood, 2000; Degenhardt et al., 2001; Von Sydow et al., 2001; for review, see Gardner and Vorel, 1998). This theory, however, has been refused by other authors who did not find any cause–effect relationships between marijuana consumption and abuse of other drugs (Hammer and Vaglum, 1990; Chen and Kandel, 1998). Only recently, this issue has been examined in laboratory animals (Gallate et al., 1999; Lamarque et al., 2001; Pontieri et al., 2001; Valverde et al., 2001; De Vries et al., 2001, 2003; Norwood et al., 2003; Solinas et al., 2003), but the results have been controversial.

In a recent study (González et al., 2004), we evaluated whether the reinforcing properties of morphine, tested in an operant progressive ratio paradigm of morphine self-administration, were different in Δ^9-THC-tolerant rats compared to controls rats. To do this, we used a chronic cannabinoid treatment that tried to be closer to the patterns of human consumption. However, our data were consistent with the notion that both Δ^9-THC-tolerant and non-tolerant rats were vulnerable to morphine to a similar extent, or, in other words, morphine was equally reinforcing for both groups of animals (González et al., 2004). Indeed, a certain tendency to self-administer less morphine in the case of Δ^9-THC-tolerant rats could be appreciated in some specific days of the analysis period (González et al., 2004). In support of this lack of differences in morphine vulnerability between Δ^9-THC-tolerant and non-tolerant rats is the fact that dopaminergic transmission in forebrain regions, which has been related to the reinforcing properties of opioids (for review, see Herz, 1998), were similar in both groups, either before morphine self-administration or after 15 days of being subjected to daily sessions of morphine access. Other studies in laboratory animals have also addressed the same question, but the results were not conclusive. Thus, some authors reported that cannabinoid pretreatment produced a behavioral sensitization to opioids (Lamarque et al., 2001; Pontieri et al., 2001; Norwood et al., 2003), and increased cocaine relapse (De Vries et al., 2001) or the motivation to drink alcohol (Gallate et al., 1999) in rats. Recently, De Vries et al. (2003) reported that cannabinoid agonists were able to reinstate heroin seeking behavior following extinction of heroin self-administration, but the authors used HU-210, a synthetic cannabinoid agonist with a extremely high pharmacological potency, strongly exceeding that of plant-derived cannabinoids, such as Δ^9-THC. Using Δ^9-THC at doses closer to those associated with the human consumption, other studies revealed, however, that the administration of this cannabinoid did not alter reinforcing properties of morphine in conditioned-place preference analysis (Valverde et al., 2001), which is in agreement with the results of our study (González et al., 2004). Based on all these data, it is possible to conclude that, although Δ^9-THC does not appear to a priori facilitate the first consumption of "stronger" drugs of abuse, the endocannabinoid system might play a role in the vulnerability, sensitization and relapse to the use of these substances in previously addicted individuals. On the other hand, two recent studies have demonstrated that CB_1 receptor antagonists might have therapeutic value in the treatment of opiate addiction (De Vries et al., 2003; Solinas et al., 2003) in addition to its well-recognized properties in the addiction to nicotine (Cohen et al., 2005) or alcohol (Colombo et al., 2005).

6. Concluding remarks and future perspectives

In the present review article, we have addressed all data existing, in experiments conducted in laboratory animals, on the behavioral and molecular bases that underly the states of tolerance, dependence and withdrawal to cannabis and cannabinoids, and these data have been compared with all information available on cannabis addiction in humans. We have provided enough evidence to assume that prolonged treatment with cannabis or selected cannabinoids produces a tolerance phenomenon for most of the pharmacological

effects of these substances. This tolerance is essentially due to pharmacodynamic responses (down-regulation/desensitization of cannabinoids receptors), although some evidence exist on pharmacokinetic effects and neuronal adaptative responses. An important issue here is that tolerance, and its associated adaptative changes in CB_1 receptors, exhibited a notable regional dependency indicating the occurrence of different mechanisms of receptor regulation, whose bases have been largely discussed. The discontinuation of chronic cannabinoid treatment does not elicit abstinence responses spontaneously in most of the studies carried out in laboratory animals, presumably because the particular pharmacokinetic characteristics of cannabinoids, but these responses may be elicited after the blockade of CB_1 receptor in cannabinoid-tolerant animals. Using these paradigms, various studies described the occurrence of withdrawal signs, that were mainly motor in nature, accompanied by changes in most of the molecular and endocrine markers affected during the abstinence to other drugs. However, the intensity of these signs was not so robust as the case of opioids, alcohol or psychostimulants. Finally, we also reviewed the data that indicate whether cannabinoid-tolerant animals might be more vulnerable to reinforcing properties of morphine, although the evidence published so far is clearly controversial for both laboratory animals and humans. By contrast, there is increasing evidence indicating that the pharmacological management of the endocannabinoid signaling might serve to treat cannabis addiction and, in particular, the addiction to other drugs such as alcohol, nicotine or opioids.

Acknowledgements

The studies mentioned in this work, which have been carried out in our laboratory, have been supported by grants from “Plan Nacional Sobre Drogas”, “Agencia Antidroga” MCYT (PM99-0056), and “Red CIEN” (C03/06). Authors are indebted to all researchers that collaborated in these experiments.

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