Signaling pathways involved in the development of cannabinoid tolerance

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Considerable plasticity exists in the endogenous cannabinoid system, as evidenced by the high degree of tolerance that develops following repetitive exposure to exogenously administered cannabinoid receptor agonists. This tolerance development is accompanied by cannabinoid CB_1 receptor downregulation and attenuation of G-protein activation. The biological processes responsible for CB_1 receptor downregulation remain to be fully understood. However, recent evidence suggests that several protein kinases participate in the development of cannabinoid tolerance. These observations implicate a role for protein kinases in cannabinoid signaling pathways. It remains to be established whether these protein kinases are directly involved in CB_1 receptor regulation or whether they contribute to tolerance by modulating additional signaling pathways.

The existence of an endogenous cannabinoid system, comprising cannabinoid CB_1 and CB_2 receptor subtypes together with their signaling pathways and endogenous ligands, is now well recognized. Although the discovery of the endogenous cannabinoid system provides an opportunity to unravel the mechanisms by which marijuana produces its complex behavioral and pharmacological effects, understanding the physiological roles of this system has even greater implications for neuroscience. Indeed, evidence is mounting that endogenous cannabinoids, such as anandamide and 2-arachadonoylglycerol, have modulatory roles in cognition, reward, appetite, pain perception, motor function, immune regulation and neuroexcitability, to name just a few putative physiological functions. Moreover, it appears that dysfunction of the endogenous cannabinoid system contributes to several pathophysiological conditions that have been associated with the abovementioned biological processes. The key to any endogenous system is its ability to adapt to maintain homeostasis. Furthermore, the degree of plasticity in any system might reflect its inherent influence on biological function. Although there is relatively little evidence demonstrating sensitization of the cannabinoid system, desensitization or tolerance to cannabinoid receptor agonists readily takes place. There is ample evidence that this tolerance is pharmacodynamic, rather than pharmacokinetic, in nature. Although only the CB_1 receptor subtype is found in neural tissue, the extent that multiple signaling pathways are modulated by this receptor subtype is unknown. At present, there is little evidence that the CB_2 receptor has a role in neuronal signaling pathways.

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Nature of cannabinoid tolerance

The acute behavioral and pharmacological effects of cannabis and its active constituent Δ^9-tetrahydrocannabinol (THC) have been well characterized and are in relatively little dispute [1]. Moreover, there is ample evidence that the magnitude of tolerance to cannabinoid receptor agonists can be considerable. Repetitive administration of THC produces tolerance to most of its pharmacological effects in dogs [2], rats [3] and mice [4]. The magnitude of tolerance varies depending on the species, agonist efficacy, treatment protocol and dependent measure. Mice injected daily with modest doses of THC for a week are 5–10 times less sensitive to a challenge dose of THC than vehicle-treated mice [5]. By contrast, a two-week treatment regimen with escalating doses of THC renders mice 50–100 times less sensitive to THC than control animals [6]. Although tolerance develops during chronic exposure to high doses of THC in humans, it is less definitive following intermittent exposure to marijuana [7]. A recent study reported relatively little tolerance to smoked marijuana or THC following three days of exposure in humans [8], whereas an earlier study reported some tolerance following a longer exposure time [9].

Tolerance represents an excellent opportunity to explore the signaling pathways within the cannabinoid system. One of the major unanswered questions is how a single receptor subtype, the CB_1 receptor, accounts for all of the centrally mediated effects of cannabinoids. It seems logical that activation of multiple signaling pathways by the CB_1 receptor contributes to this heterogeneity. Indeed, tolerance does not develop equally to all cannabinoid-mediated effects. For example, the time-course for the development of tolerance to THC-induced sedation and THC-induced analgesia differs in mice [4]. Furthermore, structurally dissimilar cannabinoid receptor agonists do not produce equivalent tolerance [10]. In a recent study, the development of tolerance to the cerebral metabolic effects of THC was shown to be regionally specific and temporally distinct [11]. In this study, the attenuation of CB_1 receptor-mediated effects on glucose metabolism closely paralleled the development of tolerance to the effects of THC, prompting these investigators to suggest

that THC-mediated effects on stress, reward and memory might persist even after prolonged exposure to THC [11]. Consistent with this observation, THC-tolerant rats exhibit an elevation in levels of the endogenous cannabinoid anandamide in limbic areas but not in other brain regions [12]. By contrast, tolerance has been demonstrated to cannabinoid-mediated modulation of synaptic currents in the nucleus accumbens, a brain region that is typically associated with the rewarding properties of THC and other psychoactive drugs [13]. Moreover, cross-tolerance was observed in this region to the synaptic effects of mu opioid receptor agonists and to endocannabinoid-mediated synaptic plasticity [13], suggesting a disruptive effect on endogenous circuitry thought to be involved in reward-induced learning [14]. Thus, cannabinoid tolerance appears to vary temporally and spatially within the CNS, and might vary between different cellular events within specific regions. Moreover, tolerance to exogenously administered cannabinoids could interfere with the functionality of the endocannabinoid system.

CB_1 receptors

The behavioral effects of cannabinoids are mediated by CB_1 receptors in the CNS. CB_1 receptors are G-protein-coupled receptors (GPCRs) that activate primarily the pertussis toxin-sensitive G proteins $G\alpha_{i/o}$ [1]. Their effector responses include inhibition of adenylyl cyclase, decreased Ca^{2+} conductance and increased K^+ conductance. Moreover, various intracellular kinases, including the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases type 1 and 2 (ERK1,2) [15,16] and p38 MAPK [17], JUN N-terminal kinase (JNK) [18], protein kinase B [PKB (also known as Akt)] [19] and focal adhesion kinase (FAK) [20], are activated following CB_1 receptor activation. CB_1 receptors are widely distributed in the CNS, with particularly high levels in: (i) the basal ganglia (caudate–putamen, globus pallidus and substantia nigra), which mediate motor function; (ii) the cerebellum, which mediates motor coordination; and (iii) the hippocampus, which is involved in memory [1].

Studies have shown that chronic administration of drugs that target GPCRs produces a series of adaptations that include uncoupling of the receptor from its G protein and decreased effector response (desensitization), sequestration into an intracellular vesicle (internalization) and either receptor degradation (downregulation) or recycling to the cell membrane [21,22]. The emerging picture is that these adaptations occur in the cannabinoid system in the CNS, although the specific mechanisms might vary by CNS region and with differing treatment paradigms. This observation is consistent with the data summarized above showing differences in tolerance to various cannabinoid-mediated behaviors.

CB_1 receptor downregulation

Receptor downregulation has been described for several GPCRs. Chronic cannabinoid treatment has been shown to reduce CB_1 receptor levels (B_{max}) in forebrain regions, including the caudate–putamen in animals rendered tolerant to cannabinoid-mediated hypomotility [23]. The existence of regional differences in CB_1 receptor downregulation was suggested by independent studies showing that: (i) no change in [^{3}H]CP55940 (a nonselective cannabinoid receptor radioligand) (see Chemical names) binding was seen in whole-brain homogenates from chronic CP55940-treated mice despite the presence of tolerance [24]; and (ii) chronic CP55940 treatment decreased CB_1 receptor binding in the striatum but not the ventral mesencephalon [25]. CB_1 receptor downregulation has been measured consistently using CB_1 receptor binding in brain sections or membrane homogenates from animals treated with THC, CP55940 or WIN552122 (a nonselective cannabinoid receptor ligand) [3,6,26,27]. Chronic administration of anandamide or the stable analog methanandamide has produced varying results, possibly because acute injection of anandamide appears to increase CB_1 receptor levels [28,29]. Autoradiographic studies have allowed analysis of a large number of brain regions and shown that downregulation occurs in all CB_1 receptor-containing brain regions, including the cerebellum, hippocampus, caudate–putamen, globus pallidus, substantia nigra, prefrontal, cingulate and entorhinal cortices, nucleus accumbens, amygdala, hypothalamus, thalamus and periaqueductal gray (PAG) [6]. However, the magnitude of downregulation varies, with a smaller change observed in the basal ganglia output nuclei: globus pallidus, entopeduncular nucleus and substantia nigra. These are also the regions of highest CB_1 receptor density, although it is not clear whether this factor is a determinant of the magnitude of downregulation. Regional differences in CB_1 receptor downregulation are accentuated at certain time points during cannabinoid treatment, as revealed by time-course studies [3,30]. Downregulation occurs more rapidly and with greater magnitude in the hippocampus and cerebellum compared with the basal ganglia. These regional differences in adaptation suggest that CB_1 receptor regulation of signaling proteins can also vary by brain region.

CB_1 receptor-activated G proteins and effectors

CB_1 receptor-mediated G-protein activation can be measured using agonist-stimulated [^{35}S]GTPγS binding in brain sections and membrane homogenates. Initial analysis of CB_1 receptor-stimulated [^{35}S]GTPγS autoradiography revealed a high level of CB_1 receptor desensitization in brains from rats that had received THC for 21 days [31]. This effect was regionally widespread, occurring in the hippocampus, cerebellum, caudate–putamen, globus pallidus, substantia nigra, septum and cortex. However, the pattern of desensitization was similar to that of downregulation, with the globus pallidus and substantia nigra exhibiting the smallest magnitude of change. Acute THC treatment did not affect CB_1 receptor-stimulated

Chemical names

CP55940: (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol
WIN552122: *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazinyl]-(1-naphthalenyl)-methanone mesylate

[^{35}S]GTPγS binding, indicating that loss of G-protein activation resulted from receptor adaptation during chronic treatment. Subsequent studies have shown that CB_1 receptor desensitization occurs after chronic administration of THC, WIN552122, CP55940 or anandamide using a variety of administration paradigms [3,6,29,32]. The regional nature of this adaptation has been revealed in several studies. Desensitization, like downregulation, develops at different rates and magnitudes in different regions [3]. The hippocampus exhibits rapid, large-magnitude desensitization; desensitization in the cerebellum develops slightly slower but to the same magnitude. By contrast, a slower rate of development and smaller magnitude of desensitization are found in the caudate–putamen and in particular the globus pallidus. Changes in G-protein mRNA have been reported and could contribute to loss of CB_1 receptor-mediated G-protein activity, but these alterations were not accompanied by concomitant changes in protein expression [33]. Thus, it is not clear whether loss of CB_1 receptor-mediated G-protein activity results from CB_1 receptor downregulation, receptor–G-protein uncoupling, altered G-protein expression or a combination of these adaptations.

Desensitization at the effector level has been more difficult to assess because of methodological limitations of the available techniques and the complexity of signaling proteins. The potential for effector desensitization is demonstrated by studies in cell culture models that have revealed desensitization of adenylyl cyclase and ERK1,2 [34]. In the CNS, chronic CP55940 treatment of mice did not alter CB_1 receptor-mediated inhibition of adenylyl cyclase in the cerebellum [35]. However, recent studies have revealed an increase in basal, forskolin-stimulated or Ca^{2+}-stimulated adenylyl cyclase activity in the cortex, striatum and cerebellum of chronic THC-treated mice [27,36,37]. Increases in the levels of cAMP are particularly pronounced in the cerebellum following antagonist-precipitated withdrawal from cannabinoids [36,37], suggesting a role for this signaling pathway in cannabinoid dependence. This phenomenon has been replicated in CB_1 receptor-transfected cell lines, where it was demonstrated that only adenylyl cyclase subtypes acutely inhibited by cannabinoid receptor agonists exhibit antagonist-precipitated enhancement of cAMP following chronic agonist treatment [38]. This adenylyl cyclase 'superactivation' effect has been described previously with other psychoactive drugs, including opiates, that inhibit adenylyl cyclase. The mechanisms that underlie this effect are unknown but could involve upregulation of specific adenylyl cyclase isoforms, increased activation of G_s and decreased activation of G_i-family G proteins [39].

Regional differences in the magnitude and temporal characteristics of downregulation and desensitization might have functional implications for the development of tolerance to various behaviors [40]. Tolerance develops to most cannabinoid-mediated behaviors, including hypothermia, hypomotility, antinociception and memory impairment, consistent with the identification of CB_1 receptor adaptation in the hypothalamus, striatum, cerebellum, PAG, spinal cord and hippocampus. The striking similarities in time-courses for the development and recovery of tolerance to specific behaviors associated with CB_1 receptor adaptation in specific regions further suggests a relationship between these events [40].

Mechanisms of CB_1 receptor adaptation

The mechanisms that regulate CB_1 receptors in the brain have not been well defined, largely because of the inherent difficulties of working with native tissue. Studies in hippocampal neuronal cultures and in a *Xenopus* oocyte expression system (exogenously expressing CB_1 receptors, K^+ channels and various regulatory proteins) have demonstrated that CB_1 receptor desensitization requires G-protein-coupled receptor kinase (GRK) and β-arrestin [41,42]. By analogy with the opioid system, where β-arrestin-2 has been implicated in morphine tolerance and mu opioid peptide receptor desensitization [43], it is logical to hypothesize that this regulatory pathway is involved in cannabinoid tolerance. Indeed, internalization of CB_1 receptors following agonist treatment has been demonstrated in CB_1 receptor-transfected cells [44] and hippocampal cultures [45], but the role of this process in adaptation in the brain is not clear. Results showing downregulation of CB_1 receptor binding sites following chronic cannabinoid treatment suggest that CB_1 receptors in the brain are internalized and degraded, but this has not yet been demonstrated directly. Moreover, because CB_1 receptors undergo a greater magnitude and more regionally widespread desensitization and downregulation than other $G_{i/o}$-coupled receptors in brain, such as mu opioid or 5-HT_{1A} receptors [46,47], it is also possible that CB_1 receptor adaptation involves a mechanism that is distinct from these other GPCRs.

Another possible explanation for decreased CB_1 receptor binding after chronic cannabinoid treatment is that receptor synthesis is decreased. It was reported that CB_1 receptor mRNA was decreased only in the caudate–putamen of animals treated with THC or CP55940 [48]. Analysis of CB_1 receptor mRNA after different durations of THC treatment showed that the magnitude and direction of change depended on the duration of treatment in addition to the interval between drug injection and sacrifice [49]. These results indicate that although alterations in CB_1 receptor synthesis might contribute to adaptation in the caudate–putamen, this is not the case in the hippocampus and cerebellum. Therefore, although substantial progress has been made in elucidating the signaling pathways used by CB_1 receptors in the naïve brain, the role of specific signaling proteins in CB_1 receptor regulation and adaptation is largely unknown.

Role of protein kinases

CB_1 receptor-mediated G-protein activation results in the generation of GTP-bound Gα and free Gβγ subunits, which both signal to effectors. Activated $G\alpha_i$ attenuates adenylyl cyclase activity leading to decreased cAMP synthesis and decreased protein kinase A (PKA) activity [1]. Depending on the adenylyl cyclase subtype, free Gβγ can either inhibit or stimulate adenylyl cyclase [50]. As described above, chronic cannabinoid administration elevates cAMP levels, which consequently increases PKA activity [32], an effect that is augmented by administration of a CB_1

receptor antagonist [36,37]. Free βγ dimers also regulate other cellular signaling events, such as ion channel conductance, phospholipid metabolism and the activity of several intracellular kinases. Cannabinoids stimulate ERK1,2 activation in cultured cell lines [15,51] and brain [16]. Interestingly, in hippocampus and neuroblastoma cells, ERK1,2 activation by cannabinoids was downstream of inhibition of the cAMP–PKA pathway [16,52]. In astrocytoma cells, however, this activation required Gβγ-sensitive phosphoinositide 3-kinase (PI3K), similar to cannabinoid activation of PKB [53]. Cannabinoids also activate p38 MAPK and JNK in several cell types [18,54], although only p38 was found to be activated in hippocampus [17]. There is also evidence for cannabinoid activation of non-receptor tyrosine kinases in the brain, including a neuronal FAK isoform that was activated via recruitment of the Src family kinase Fyn [55]. Similar to ERK1,2 activation, evidence suggested that these signaling events were a result of inhibition of cAMP–PKA signaling.

Although it is well known that plasticity of an endogenous system often occurs via phosphorylation events, only recently have there been attempts to demonstrate a role for protein kinases in tolerance development. A recent study demonstrated that Src tyrosine kinase and PKA are involved in tolerance to spinally mediated cannabinoid-mediated analgesia [56]. Tolerance to THC was reversed with either a Src family tyrosine kinase inhibitor or a PKA inhibitor. Inhibitors of PKC, PKG and PI3K were ineffective in altering tolerance to this effect, despite an earlier report that PKC activation disrupted CB_1 receptor signaling through direct phosphorylation of the receptor [57]. None of the inhibitors influenced the acute analgesic effects of THC. These studies were followed up with attempts to determine whether these kinases were involved to the same extent in the development of tolerance to other cannabinoid effects. Inhibitors of PKA, PKG, PKC and Src tyrosine kinase have been shown to have no influence on the acute effects of THC in altering spontaneous activity, pain sensitivity, body temperature and producing catalepsy (C. Bass and B.R. Martin, unpublished). Moreover, inhibitors of PKG and PKC had no influence on the development of tolerance to any of these effects. By contrast, inhibitors of PKA and Src tyrosine kinase reversed tolerance to some, but not all, effects of THC. Specifically, the Src tyrosine kinase inhibitor reversed tolerance to the sedative effects of THC but not to any of the other pharmacological effects, including analgesia. The PKA inhibitor reversed tolerance to all of THC effects. Taken together, these data suggest that PKA activity has a major role in THC-induced tolerance for at least three measures of mouse behavior, and that Src family kinases contribute to tolerance to a subset of these effects. If direct involvement of specific protein kinases in cannabinoid tolerance is confirmed, future studies will need to determine which targets of these protein kinases mediate this effect.

Conclusions and perspectives

Chronic cannabinoid administration produces tolerance to many cannabinoid effects, including sedation, analgesia, hypothermia, catalepsy and impairment of short-term memory. In animal models, this tolerance is associated with both downregulation of CB_1 receptors and desensitization of CB_1 receptor-mediated G-protein activation. These adaptive responses occur in all brain regions that express cannabinoid receptors but there is regional variability in the rate and magnitude of the adaptive response. Some evidence suggests that regional variation in CB_1 receptor adaptation might underlie differential tolerance to cannabinoid-mediated pharmacological

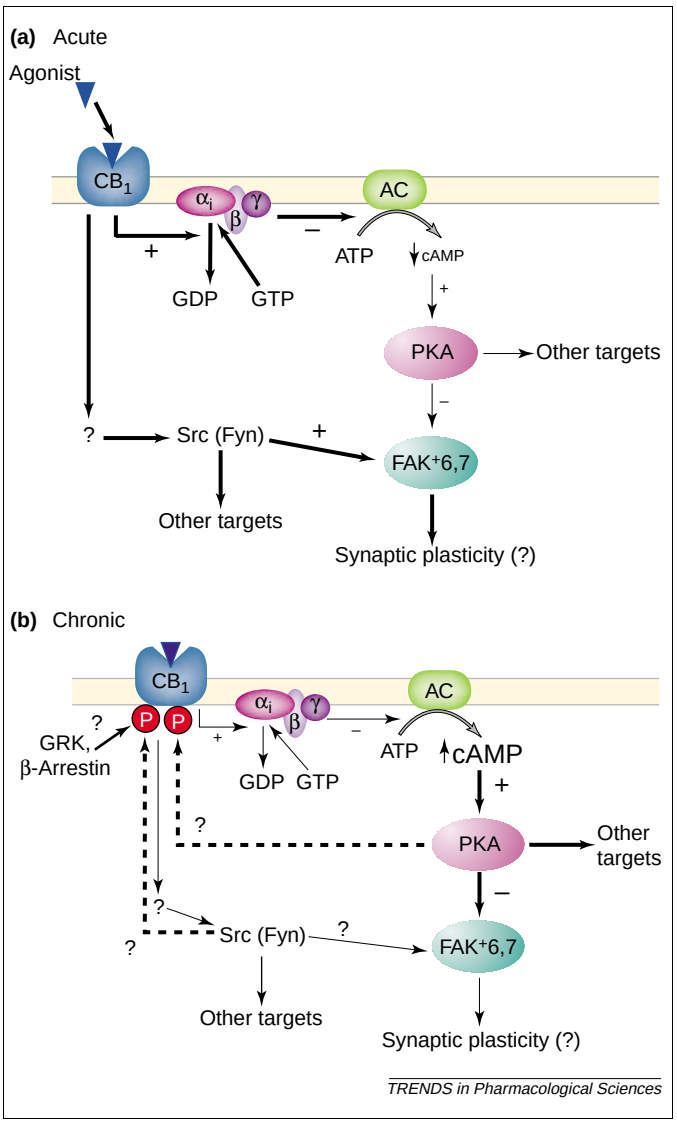

Figure 1. Cannabinoid CB_1 receptor signaling pathways implicated in cannabinoid tolerance. **(a)** Acute: agonist-mediated activation of the CB_1 receptor stimulates guanine nucleotide exchange to activate $G_{i/o}$ proteins. The GTP-bound Gα, alone or along with free βγ subunits of the G protein, inhibit adenylyl cyclase activity. The resulting decrease in cAMP levels results in attenuation of protein kinase A (PKA) activity. This has various consequences, including the disinhibition of a neuronal focal adhesion kinase (FAK) isoform. CB_1 receptor activation might lead to Src family tyrosine kinases via an unknown mechanism, and this contributes to FAK activation and potential effects on synaptic plasticity. **(b)** Chronic: persistent exposure to the agonist leads to uncoupling of the CB_1 receptor from $G_{i/o}$, possibly via G-protein-coupled receptor kinase (GRK)–arrestin-mediated desensitization and downregulation. The levels of cAMP are also elevated, possibly through rebound hyperactivation of adenylyl cyclase, which results in increased PKA activity. PKA then phosphorylates target proteins that are involved in the maintenance of cannabinoid tolerance, possibly including the CB_1 receptor itself. Presumably, FAK activity would also be inhibited by increased PKA, and any CB_1 receptor influence on Src kinase activity would be diminished. However, Src family kinases might have an as yet unknown role in maintaining cannabinoid tolerance.

effects. By analogy with cultured cell models, the mechanisms of CB_1 receptor desensitization and downregulation might include GRK-mediated phosphorylation and association of the receptor with β-arrestin, although this remains to be established in the CNS. Changes in expression of the gene encoding the CB_1 receptor might also play a role in some brain regions, but not others. Another cellular consequence of chronic cannabinoid administration is a compensatory increase in cAMP synthesis with concomitant enhancement of PKA activity (Figure 1). This adaptive response also exhibits regional variability and is most evident following administration of a CB_1 receptor antagonist. In support of a role of upregulated cAMP signaling in cannabinoid tolerance, administration of a PKA inhibitor into the CNS reverses the diminution in responsiveness to cannabinoids that occurs following chronic administration. It appears that PKA phosphorylates target proteins that are involved in the maintenance of tolerance, possibly including the CB_1 receptor itself. Similar reversal of tolerance to a subset of cannabinoid-induced effects has been observed with a Src family tyrosine kinase inhibitor. However, tolerance reversal was not produced by inhibitors of PKB, PKC or PKG. These results suggest specific involvement of PKA and Src-family kinase pathways in cannabinoid tolerance, but further research is needed to confirm and define their role in the manifestation of tolerance. Furthermore, it remains to be determined whether these kinases contribute to CB_1 receptor desensitization and downregulation or whether they have a distinct role in the development or maintenance of cannabinoid tolerance.

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