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Changes in the Expression of G Protein-Coupled Receptor Kinases and β-Arrestins in Mouse Brain During Cannabinoid Tolerance

A Role for Ras–ERK Cascade

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Abstract

The focus of our study was to determine the role of G protein-coupled receptor kinases (GRKs) and β-arrestins in agonist-induced CB1 receptor modulation during cannabinoid tolerance and their dependence from the extracellular signal-regulated kinase (ERK) cascade. In wild-type mice, chronic Δ^9-tetrahydrocannabinol (THC) exposure significantly activated specific GRK and β-arrestin subunits in all the considered brain areas (striatum, cerebellum, hippocampus, and prefrontal cortex), suggesting their involvement in the adaptive processes underlying CB1 receptor downregulation and desensitization. These events were ERK-dependent in the striatum and cerebellum, because they were prevented in the genetic (Ras-GRF1 knockout mice) and pharmacological (SL327-pretreated mice) models of ERK activation inhibition, whereas in the hippocampus and prefrontal cortex, they appeared to be mostly ERK-independent. In the latter areas, ERK activation after chronic THC increased the transcription factors cyclic adenosine monophosphate response element-binding protein and Fos B as well as a downstream protein known as brain-derived neurotrophic factor. As a whole, our data suggest that in the striatum and cerebellum, THC-induced ERK activation could represent a key signaling event to initiate homologous desensitization of CB1 receptor, accounting for the development of tolerance to THC-induced hypolocomotion. In the prefrontal cortex and hippocampus, THC-induced alteration in GRKs and

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β-arrestins primarily depends on other kinases, whereas ERK activation could be part of the molecular adaptations that underlie the complex behavioral phenotype that defines the addicted state.

Index Entries: Cannabinoid tolerance; GRKs; β-arrestins; ERK pathway; CB1 receptor regulation; Ras-GRF1 knockout mice; SL327.

Introduction

Repeated exposure to Δ^9-tetrahydrocannabinol (THC), the psychoactive ingredient in marijuana, results in the development of tolerance to most acute behavioral and pharmacological effects *(1)*. Several studies have attempted to identify the cellular adaptations underlying the development of tolerance to THC, and all results have suggested that the CB1 receptor plasticity plays a major role *(2)*. Studies in cell culture models have shown that chronic exposure of CB1 receptors to cannabinoid agonists produces CB1 receptor downregulation and desensitization. Similarly, chronic administration of cannabinoids in animals has shown that these same events occur in the central nervous system *(3–10)*; however, the mechanisms that regulate CB1 receptors in the brain are not well defined.

Recent works have suggested a significant involvement of protein kinases in tolerance development to THC. Lee et al. *(11)* demonstrated that Src tyrosine kinase and protein kinase A were involved in tolerance to spinally mediated cannabinoid analgesia because it was reversed with either a Src family tyrosine kinase inhibitor or a protein kinase A inhibitor. The involvement of the extracellular signal-regulated kinase (ERK) pathway in the phenomena triggered by chronic cannabinoid exposure was demonstrated in studies in which genetic and pharmacological inhibition of THC-induced ERK activation was applied *(12,13)*. The genetic approach involved Ras-specific guanine nucleotide exchange factor (Ras-GRF1) knockout mice, whereas the pharmacological model used the compound SL327, a drug that crosses the blood–brain barrier and prevents the activation of ERK by inhibiting mitogen-activated protein kinase/extracellular signal-regulated kinase-activating kinase, the upstream kinase of ERK *(14,15)*. THC-induced ERK activation did not occur in either model *(12,13)*.

Ras–ERK signaling was not necessary for triggering the acute effect of THC: wild-type, knockout, and SL327-pretreated wild-type animals presented significant cannabinoid-induced hypomotility. When THC was administered chronically, only wild-type animals developed full tolerance to this effect, whereas in both knockout and SL327-pretreated mice there was a significant reduction in spontaneous locomotor activity.

The impact of the inhibition of ERK activation on the biological processes involved in cannabinoid tolerance (receptor downregulation and desensitization) was then surveyed. In the caudate-putamen and cerebellum of Ras-GRF1 knockout mice and SL327-pretreated wild-type mice, CB1 receptor adaptations did not occur, suggesting that ERK activation might account for CB1 receptor plasticity involved in the development of tolerance to THC hypolocomotor effect. Conversely, the hippocampus and prefrontal cortex showed CB1 receptor adaptations, regardless of the genetic or pharmacological inhibition of the ERK pathway, suggesting regional variability in the cellular events underlying the altered CB1 receptor function *(13)*. To further describe the role of the Ras–ERK pathway in the events triggered by chronic THC, the issues that must now be addressed regard the mechanism linking the ERK pathway with CB1 receptor regulation in the striatum and cerebellum and the cellular events triggered by ERK activation in the hippocampus and prefrontal cortex, where this pathway is not directly

involved in CB1 receptor plasticity. We addressed these questions by using the genetic and pharmacological models in which THC-induced ERK activation is lost. The first aim was to determine the possible role for G protein-coupled receptor kinases (GRKs) and β-arrestins in CB1 receptor function regulation in the striatum and cerebellum and their dependence on the Ras–ERK pathway. Second, because the prominent role of ERK signaling is to alter gene expression through modulation of different transcription factors, we investigated the role of ERK in modulating phosphorylated cyclic adenosine monophosphate response element-binding protein (pCREB), Fos B, and a downstream protein (brain-derived neurotrophic factor [BDNF]) in the hippocampus and prefrontal cortex after in vivo THC.

Materials and Methods

Drugs

THC (a generous gift from GW Pharmaceutical, Salisbury, United Kingdom) was dissolved in ethanol, cremophor, saline (1:1:18).

SL327 (a gift from Dr. Trzaskos, Bristol-Myers Squibb Co., Princeton, NJ) was dissolved in dimethyl sulfoxide.

Animals and Treatment

Ras-GRF1 +/+ and −/− mice originating from the leading strain characterized by Brambilla et al. *(16)* were housed (three per cage) in a controlled environment at constant temperature and humidity on a 12-h light–dark cycle, with free access to food and water. They were acutely treated with THC or its vehicle subcutaneously at a dose of 10 mg/kg; for the chronic study, mice were injected with THC (10 mg/kg) or vehicle twice a day for 4.5 d. This is a well-known chronic treatment protocol inducing tolerance in mice *(12,17)*.

For the chronic studies using SL327, 1 h before every injection of THC, wild-type mice were subcutaneously administered 50 mg/kg of the compound. Experiments were performed in strict accordance with the guidelines released by the Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B) and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize both the number of animals used and their suffering.

Biochemical Studies

Thirty minutes after the last THC injection, mouse brains were quickly removed and the cerebral areas (prefrontal cortex, striatum, hippocampus, cerebellum) were obtained within a few minutes by regional dissection on ice and were immediately frozen in liquid nitrogen and stored at –80°C until they were used.

Tissue Preparation

Each brain region was homogenized in an appropriate volume of ice-cold Buffer A (10 m*M* HEPES pH 7.5, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 2 m*M* dithiothreitol, 1 m*M* PMSF, 1 m*M* EDTA, 1 m*M* EGTA, 2 m*M* sodium orthovanadate, 50 m*M* NaF, 10 m*M* sodium pyrophosfate, 0.5 % Triton, 5 mg/mL of aprotinin, and 5 mg/mL of leupeptin) and centrifuged at 11,500*g* at 4°C for 3 min. The supernatant was used as total lysate or for cytosolic and membrane preparation, and pellet was used for nuclear extracts. For the cytosolic preparation, the supernatant was saved and centrifuged at 40,000*g* at 4°C for 20 min, and the resulting supernatant was used as soluble fraction. For membrane preparations, pellets from the 40,000*g* centrifugation were resuspended in an appropriate volume of ice-cold Buffer C (20 m*M* HEPES, pH 7.5, 400 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10 m*M* NaF, 10 μ*M* Na_2MnO_4, 0.1 m*M* EDTA, 1 m*M* sodium orthovanadate, 10 m*M* PNPP, 10 m*M* β-glycerophosphate, 20% glycerol, 2 m*M* dithiothreitol, and protease inhibitors [as mentioned earlier]). A solubilization buffer (HEPES 10 m*M*, 8% sodium dodecyl sulfate) was then added in the ratio 0.2:1 vs Buffer C, and the solution was sonicated on ice

for 10 s. For nuclear extracts preparation, the pellet was resuspended in an appropriate volume of ice-cold Buffer C and was homogenized. After 30 min of incubation on ice with gentle rocking, samples were centrifuged at 11,500*g* at 4°C for 10 min, and the resulting supernatant was saved and used as nuclear extract. Protein concentrations in the respective fractions were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL).

Western Blotting

GRKs and β-arrestins were detected in different cellular fractions depending on their features. Specifically, GRK2 was detected in both cytosolic and membrane fraction, and the other GRKs were detected in total lysates and β-arrestins in membrane. Equivalent amounts of protein from the different fractions for each sample were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted electrophoretically to nitrocellulose membrane, blocked, and then incubated overnight with polyclonal or monoclonal antibodies that selectively recognized GRK2, GRK4, GRK5, GRK6, β-arrestin-2 (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:100), and phosphorylated β-arrestin-1 (Biosource, Nivelles, Belgium; diluted 1:2000). Bound antibodies were detected with horseradish peroxidase-linked antibodies (Santa Cruz Biotechnology; diluted 1:2000–5000) and were developed using an enhanced chemiluminescence reagent (Amersham Biosciences, Milan, Italy). The relevant immunoreactive bands were quantified with scanning densitometry using Scion Image software. To allow comparison between different autoradiographical films, the density of the bands was expressed as a percentage of the average of controls.

Transcription Factors Evaluation

The level of activated transcription factors were measured in the nuclear extracts using transcription factor assay kits (Active Motif, Rixensart, Belgium) based on enzyme-linked immunosorbent assay methods to detect and quantify pCREB and FosB activation. Briefly, oligonucleotides containing a cAMP-responsive element or a TPA-responsive element were immobilized in a 96-well plate. CREB or AP-1 dimers contained in nuclear cell extracts bound specifically to these oligonucleotides. By using an antibody directed against pCREB or Fos B, the equivalent transcription factors bound to the oligonucleotide were detected. Addition of a secondary antibody conjugated to horseradish peroxidase provided sensitive colorimetric readout that was quantified by spectrophotometry at 450 nm.

BDNF Evaluation

The levels of BDNF in the prefrontal cortex and hippocampus of mice were determined by a sandwich enzyme immunoassay kit (Chemicon International, Temecula, CA). Briefly, rabbit polyclonal antibodies generated against BDNF were coated onto a microplate and used to capture BDNF from the sample. BDNF-specific, biotin-conjugated, mouse monoclonal antibodies detected the captured BDNF. After addition of streptavidin-enzyme, substrate, and stop solution, the amount of BDNF was determined by reading the plate at 450 nm.

Statistical Analysis

The data presented in Figs. 1–6 are the means ±SEM of at least five animals. Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test performed by Prism (GraphPad Software, San Diego, CA).

Results

Effect of THC Treatment on GRKs and β-Arrestins in the Genetic Model

The lack of CB1 receptor downregulation and desensitization in the striatum and cerebellum of Ras-GRF1 knockout mice following

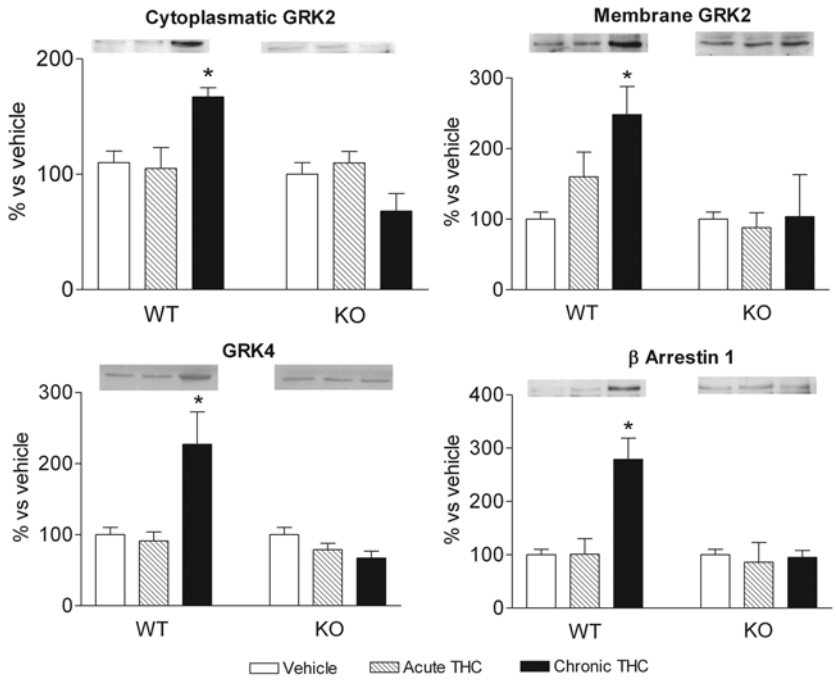

Fig. 1. Effect of THC treatment on GRK and β-arrestin levels in the striatum of wild-type and Ras-GRF1 knockout mice. Proteins from the striata of vehicle, acute (10 mg/kg subcutaneously), or chronic (10 mg/kg subcutaneously twice a day for 4.5 d) THC-treated wild-type and knockout mice (prepared as described in the Materials and Methods section) were subjected to immunoblot analysis using antibodies specific for the different GRK and β-arrestin subunits. Among all the trafficking proteins checked, only GRK2, GRK4, and β-arrestin-1 were altered by THC in wild-type mice. Data correspond to means ±SEM of at least four animals. Statistical analysis was performed with ANOVA followed by Bonferroni. *$p < 0.05$ vs vehicle.

chronic THC treatment *(13)* prompted us to investigate the involvement of the trafficking proteins in this event. Based on the model proposed for G protein-coupled receptor regulation by Lefkowitz et al. *(18,19)* and on the fact that a GRK and β-arrestin produced CB1 receptor desensitization in the *Xenopus* oocyte expression system *(20)*, we checked the immunoreactivity levels of the different GRKs predominantly expressed in the central nervous system (i.e., GRK2, -4, -5, and -6) and the two β-arrestins (i.e., β-arrestin-1 and -2) in the striatum and cerebellum of Ras-GRF1 wild-type and knockout mice after in vivo THC treatment.

Among all the GRK subunits tested in the striatum of wild-type mice, chronic THC treatment significantly increased the level of GRK2 and GRK4 (Fig. 1). Conversely from GRK4, which is already anchored to the membrane, GRK2 is located mainly in the cytosolic fraction, but the plasma membrane represents a site for its action; therefore, we wanted to determine whether GRK2 is also translocated to the membrane compartment. We detected a significant increase in GRK2 immunoreactivity in plasma membrane of wild-type mice, suggesting its membrane translocation following chronic THC treatment (Fig. 1). Finally, chronic THC treatment produced a significant raise in the immunoreactivity level of phosphorylated β-arrestin-1 (the active form) in wild-type mice. This suggests that GRK2, GRK4, and β-arrestin-1 could cooperate to promote CB1 receptor downregulation and desensitization

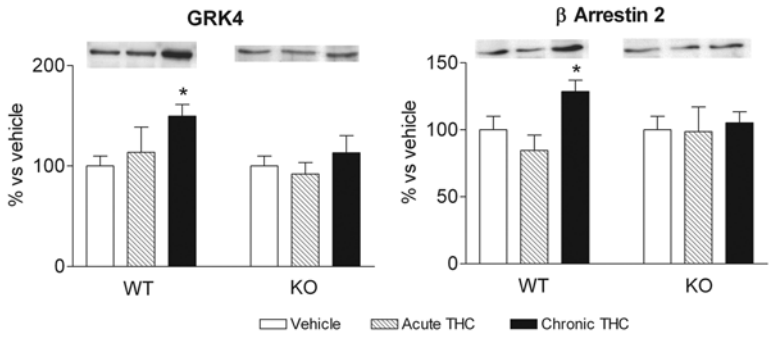

Fig. 2. Effect of THC treatment on GRK and β-arrestin levels in the cerebellum of wild-type and Ras-GRF1 knockout mice. Proteins from the cerebella of vehicle, acute (10 mg/kg sc), or chronic (10 mg/kg subcutaneously, twice a day for 4.5 d) THC-treated wild-type and knockout mice (prepared as described in the Materials and Methods section) were subjected to immunoblot analysis using antibodies specific for the different GRK and β-arrestin subunits. Among all the trafficking proteins checked, only GRK4 and β-arrestin-2 were altered by THC in wild-type mice. Data correspond to means ±SEM of at least four animals. Statistical analysis was performed with ANOVA followed by Bonferroni. *$p < 0.05$ vs vehicle.

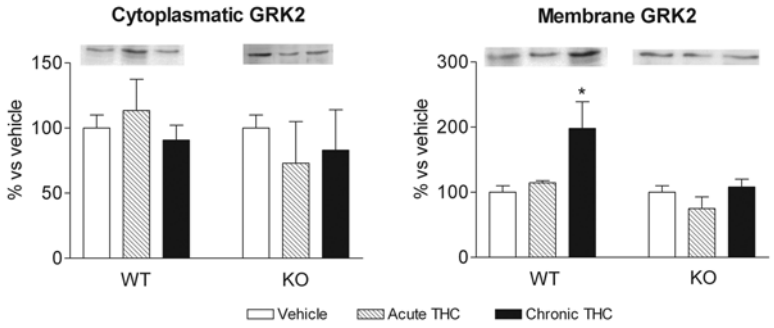

Fig. 3. Effect of THC treatment on GRK and β-arrestin levels in the prefrontal cortex of wild-type and Ras-GRF1 knockout mice. Proteins from the prefrontal cortices of vehicle, acute (10 mg/kg subcutaneously), or chronic (10 mg/kg subcutaneously twice a day for 4.5 d) THC-treated wild-type and knockout mice (prepared as described in the Materials and Methods section) were subjected to immunoblot analysis using antibodies specific for the different GRK and β-arrestin subunits. Among all the trafficking proteins checked, only membrane-associated GRK2 was altered by THC in wild-type mice. Data correspond to means ±SEM of at least four animals. Statistical analysis was performed with ANOVA followed by Bonferroni. *$p < 0.05$ vs vehicle.

in the striatum of wild-type mice. All of these events did not occur in knockout mice (Fig. 1), in which CB1 receptor function was not modified by chronic THC treatment *(13)*.

Among all the trafficking proteins tested in the cerebellum of wild-type mice, chronic THC treatment significantly increased the level of GRK4 and β-arrestin-2 (Fig. 2). Concerning β-arrestin-2, the only commercially available antibodies are raised against the nonphosphorylated form of the protein, preventing us from distinguishing between the active (phosphorylated) and inactive forms; however, its presence in membrane could mean that β-arrestin-2 is in the active form. Together, these data suggest that GRK4 and β-arrestin-2 could be involved in triggering CB1 receptor modulation in this area. As observed in the striatum, Ras-GRF1 knockout mice that did not develop CB1 receptor downregulation and

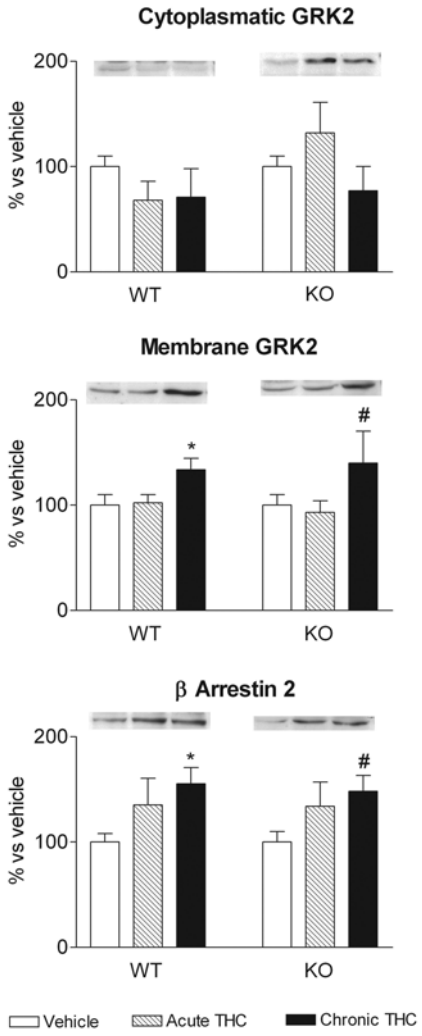

Fig. 4. Effect of THC treatment on GRK and β-arrestin levels in the hippocampus of wild-type and Ras-GRF1 knockout mice. Proteins from the hippocampi of vehicle, acute (10 mg/kg subcutaneously), or chronic (10 mg/kg subcutaneously twice a day for 4.5 d) THC-treated wild-type and knockout mice (prepared as described in the Materials and Methods section) were subjected to immunoblot analysis using antibodies specific for the different GRK and β-arrestin subunits. Among all the trafficking proteins checked, only GRK2 and β-arrestin-2 were altered by THC in wild-type mice. Data correspond to means ±SEM of at least four animals. Statistical analysis was performed with ANOVA followed by Bonferroni. *$p < 0.05$ vs wild-type vehicle; #$p < 0.05$ vs knockout vehicle.

desensitization *(13)* did not show any alteration in either GRK4 or β-arrestin-2 levels (Fig. 2).

These results suggest that the Ras–ERK cascade plays a pivotal role in CB1 receptor plasticity in the striatum and cerebellum.

The involvement of trafficking proteins in CB1 receptor adaptations after in vivo chronic exposure was also surveyed in the prefrontal cortex and hippocampus of wild-type and knockout mice. As shown in Fig. 3, in the prefrontal cortex, chronic THC significantly increased GRK2 levels in plasma membranes of wild-type mice without affecting the cytosolic fraction, whereas β-arrestin subunits were not affected (data not shown). No increases of GRK2 were observed in Ras-GRF1 knockout mice (Fig. 3). Finally, there was a significant increase of membrane-associated GRK2 and β-arrestin-2 in the hippocampus of both genetic strains (Fig. 4), suggesting that these events are independent of Ras–ERK.

Effect of THC Treatment on GRKs and β-Arrestins in the Pharmacological Model

To validate the role of the ERK pathway in the modulation of trafficking proteins induced by chronic THC, we confirmed this phenomenon in the pharmacological model. The results on trafficking proteins after the pharmacological inhibition of ERK activation require some discussion. SL327 pretreatment did not significantly affect the basal level of GRKs and β-arrestins in any of the considered brain areas (Table 1); however, chronic THC treatment altered their immunoreactivity differently, depending on the brain region considered. Similarly to knockout animals, in the striatum of SL327-pretreated mice, GRK2 and β-arrestin-1 levels were not affected by chronic treatment, whereas GRK4 immunoreactivity was decreased (80% $p < 0.05$ vs SL + vehicle; Table 1). Similarly, in the cerebellum of SL327-pretreated mice, chronic THC treatment did not affect either GRK4 or β-arrestin-2 levels (Table 1), thus confirming the results obtained in the genetic model. In the prefrontal cortex, SL327 pretreatment not only prevented increase of GRK2 but

Table 1
Effect of THC on GRK and β-Arrestin Levels
in Different Cerebral Areas of SL327-Pretreated Wild-Type Mice

Brain area	Treatment	GRK2	GRK4	p-β-arrestin-1	β-arrestin-2
		% Control			
Striatum	Vehicle	100	100	100	
	SL + vehicle	116 ± 18	120 ± 12	78 ± 13	
	Chronic THC	248 ± 40[a]	227 ± 36[a]	279 ± 40[a]	NA
	SL + chronic THC	95 ± 7	130 ± 6[b,c]	118 ± 10	
Cerebellum	Vehicle	100		100	
	SL + vehicle	NA	116 ± 11	NA	84 ± 11
	Chronic THC		150 ± 11[a]		129 ± 9[a]
	SL + chronic THC		120 ± 10		80 ± 18
Hippocampus	Vehicle	100		100	
	SL + vehicle	101 ± 8	NA	NA	104 ± 8
	Chronic THC	134 ± 11[a]			155 ± 15[a]
	SL + chronic THC	130 ± 12[a]			165 ± 20[a]
Prefrontal cortex	Vehicle	100			
	SL + vehicle	97 ± 5	NA	NA	NA
	Chronic THC	198 ± 21[a]			
	SL + chronic THC	37 ± 16[b]			

[a] $p < 0.05$ vs vehicle.
[b] $p < 0.01$ vs chronic THC.
[c] $p < 0.05$ vs SL327 + vehicle.

Proteins from the cerebral areas of mice pretreated with SL327 (50 mg/kg subcutaneously 1 h before every THC injection) that were then administered chronic vehicle or THC (10 mg/kg subcutaneously twice a day for 4.5 d), prepared as described in the Materials and Methods section. They were then subjected to immunoblot analysis using antibodies specific for the different GRK and β-arrestin subunits. Data correspond to means ±SEM of at least four animals. Statistical analysis was done with ANOVA followed by Bonferroni. THC, Δ^9-tetrahydrocannabinol; GRK, G protein-coupled receptor kinases; NA, no significant alteration in any experimental group.

even reduced it about 70% (Table 1). Finally, in the hippocampus, SL327 pretreatment did not prevent increases in GRK2 and β-arrestin-2, further confirming that in this area, CB1 receptor plasticity is independent of ERK (Table 1).

Role of the Ras–ERK Pathway in Transcription Factors Activation After Chronic THC Treatment

Because the Ras–ERK cascade appears to not be mainly involved in CB1 receptor plasticity in the hippocampus and prefrontal cortex (present work and ref. *13*), we decided to investigate whether its prominent role in these brain regions could lie in altering gene expression through modulation of different transcription factors. Therefore, we checked THC-induced alteration in CREB and Fos B activity, as well as their downstream protein BDNF, in the genetic and pharmacological models.

In the genetic model, pCREB and Fos B levels were significantly elevated both in the prefrontal cortex and hippocampus of wild-type mice following chronic THC treatment (Fig. 5). This increase did not occur in Ras-GRF1 knockout mice (Fig. 5). The downstream protein BDNF presented the same course in the hippocampus (i.e., increase in wild-type mice and no alteration in knockout animals), whereas in the prefrontal cortex, neither wild-type nor knockout mice showed significant alterations (Fig. 5).

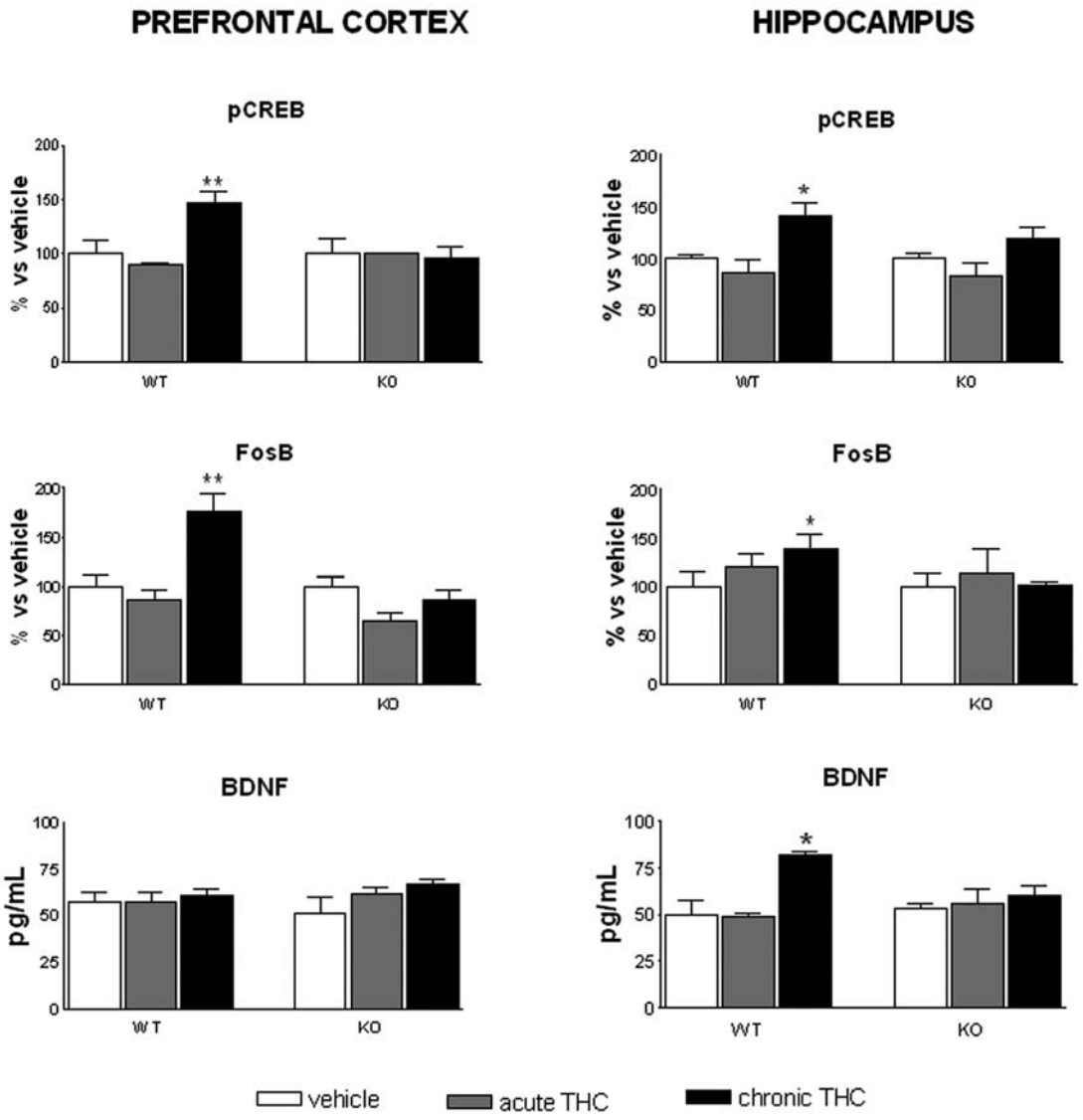

Fig. 5. Effect of THC on pCREB, Fos B, and BDNF in the prefrontal cortex and hippocampus of wild type and Ras-GRF1 knockout mice. Nuclear extracts from the cerebral areas of vehicle, acute (10 mg/kg subcutaneously), or chronic (10 mg/kg subcutaneously twice a day for 4.5 d) THC-treated wild-type and knockout mice were used to evaluate pCREB and Fos B using transcription factor assay kits based on ELISA method, whereas cellular lysates were used to measure the amount of BDNF through a sandwich enzyme immunoassay (as described in the Materials and Methods section). Data correspond to means ±SEM of at least four animals. Statistical analysis was performed with ANOVA followed by Bonferroni. $*p < 0.05$; $**p < 0.01$ vs vehicle.

Finally, SL327 pretreatment had the same impact on transcription factors modulation as it had in Ras-GRF1 knockout mice—namely, inhibition of pCREB and Fos B increases in the prefrontal cortex and hippocampus (Fig. 6). As observed in the genetic model, in the prefrontal cortex, THC did not alter BDNF levels, regardless of the pharmacological treatment

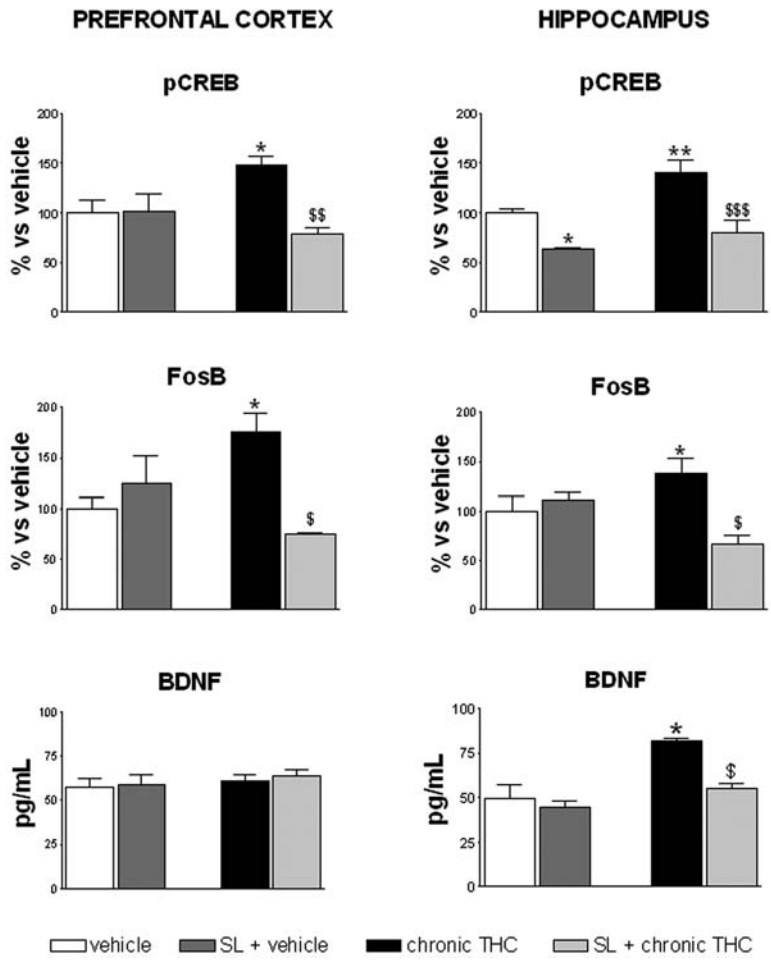

Fig. 6. Effect of THC on pCREB, Fos B, and BDNF in the prefrontal cortex and hippocampus of SL327-pretreated wild-type mice. Nuclear extracts from the cerebral areas of mice pretreated with SL327 (50 mg/kg subcutaneously 1 h before every THC injection) and then administered with chronic vehicle or THC (10 mg/kg subcutaneously twice a day for 4.5 d) were used to evaluate pCREB and Fos B using transcription factor assay kits based on enzyme-linked immunosorbent assay method, whereas cellular lysates were used to measure the amount of BDNF through a sandwich enzyme immunoassay (as described in the Materials and Methods section). Data correspond to means ±SEM of at least four animals. Statistical analysis was performed with ANOVA followed by Bonferroni. *$p < 0.05$; **$p < 0.01$ vs vehicle; \$$p < 0.05$; \$\$$p < 0.01$; \$\$\$$p < 0.001$ vs chronic THC.

(Fig. 6), whereas in the hippocampus, pretreatment with SL327 prevented increases in BDNF induced by THC (Fig. 6).

Discussion

This study provides new insight on the modulation of GRKs and β-arrestins in mouse brain during cannabinoid tolerance and their dependence from the Ras–ERK pathway.

Similarly to most G protein-coupled receptors, the CB1 receptor undergoes to desensitization after continued exposure to an agonist event that partakes in the molecular phenomena underlying the onset of cannabinoid tolerance (for review, *see* ref. 2). Desensitization of G protein-coupled receptors is often associated

with phosphorylation of the receptor by GRKs followed by binding of β-arrestin, which promotes not only the uncoupling of the receptor from G proteins but also targeting to clathrin-coated pits for internalization *(21)*. In the *Xenopus* oocyte expression system, Jin et al. *(20)* demonstrated that agonist-dependent CB1 receptor desensitization was effectively mediated by GRKs and β-arrestins; however, no studies in living animals dealt with the functional relationship between activation of CB1 receptor and modulation of trafficking proteins. Our data in wild-type mice appear to suggest that alterations in the level of GRKs and β-arrestins might be involved in long-term adaptive changes (i.e., downregulation and desensitization) in CB1 receptor activity, contributing to tolerance development in living animals. The observed alterations appear to be region-dependent: in the striatum, chronic THC significantly upregulated GRK2, GRK4, and β-arrestin-1, suggesting that these proteins can contribute to CB1 receptor downregulation and desensitization in this area. Moreover, GRK2 levels were increased in the cytosolic- and membrane-associated fraction, suggesting a regulation of both GRK2 expression (cytosolic levels) and activity (translocated amount).

In the cerebellum, GRK4 and β-arrestin-2 appear to be involved in THC-induced CB1 receptor regulation, whereas GRK2 and β-arrestin-2 play a role in the hippocampus. Finally, in the prefrontal cortex, only upregulation of membrane-associated GRK2 was observed. These results confirm previous data obtained in vitro *(20)* and may add new insight into the mechanisms of CB1 receptor regulation in vivo.

First, different trafficking proteins are involved in THC-induced CB1 receptor adaptation, depending on the brain region. The specific regional differences in the relative contribution of each GRK or β-arrestin to the chronic receptor regulation might reflect differences in the trafficking machinery between neuronal populations, which could account for the diverse time-course or magnitude in CB1 receptor plasticity observed in distinct brain regions *(7,22)*. A region-dependent increase in GRKs and β-arrestins in living animals was already associated with modulation of μ-opioid receptor signaling in models of opioid tolerance *(23)*. The same molecular scheme may now be extended to CB1 receptor, another G protein-coupled receptor, suggesting that it represents a common regulatory mechanism for several members of this superfamily.

Second, regional selectivity was also observed about the Ras–ERK dependency of the trafficking protein modulation by THC: striatum and cerebellum showed ERK-dependent events, whereas hippocampus did not. Specifically, in Ras-GRF1 knockout mice as well as in SL327-pretreated wild-type animals, chronic THC treatment did not induce any significant alteration in levels of GRKs and β-arrestins in the striatum and cerebellum, clearly suggesting the involvement of the Ras–ERK kinase cascade in these events. In our previous work *(13)*, we demonstrated that in Ras-GRF1 knockout and SL327-pretreated mice, THC chronic in vivo treatment did not induce either CB1 receptor downregulation or desensitization in these brain areas.

Together, we can speculate on the ERK-dependent mechanisms involved in CB1 receptor regulation in the striatum and cerebellum. Among the different signaling events triggered by CB1 receptor stimulation, in these cerebral areas, THC triggers ERK activation that, in turn, leads to CB1 receptor phosphorylation by GRKs (as demonstrated by Jin et al. *[20]* in an in vitro expression system), thereby promoting β-arrestin association and then desensitization and internalization. In this situation, ERK activation represents an initial event in CB1 receptor function regulation, and does not require functional β-arrestin and dynamin like other G protein-coupled receptors *(24,25)* where receptor endocytosis plays an important role in the assembly of signaling proteins involved in ERK activation. Indeed, the requirement of receptor endocytosis for ERK activation does not appear to be a general property of all G protein-coupled receptors nor all cellular systems. For example, for A_3 adenosine receptor

(26) and μ-opioid receptor *(27)*, studies have demonstrated that ERK recruitment is a key signaling event for initiation of homologous desensitization. According to this view, CB1 receptor desensitization in the striatum and cerebellum may represent another example of ERK-dependent homologous desensitization.

A different picture emerges for the hippocampus. In this region, trafficking proteins (GRK2 and β-arrestin-2) were altered by THC treatment in the same manner in wild-type and knockout mice as well as in SL327-pretreated animals, suggesting that these events occurred independently by the ERK activation. These results are consistent with our previous data *(13)*, which show the presence of CB1 receptor desensitization and downregulation after chronic THC in Ras-GRF1 knockout and SL327-pretreated mice, and strengthen the hypothesis that other protein kinases are involved in CB1 receptor plasticity in the hippocampus. Furthermore, in this cerebral area, GRK2 levels were increased only in the membrane-associated fraction of all experimental groups (wild-type, knockout, and SL327-pretreated mice), suggesting a modulation of GRK2 activation without changes in its expression.

Finally, in the prefrontal cortex, the prevention of ERK activation in both models blocked membrane-associated increases in GRK2. In our previous work *(13)*, different CB1 receptor adaptation was shown following chronic THC treatment in the genetic and pharmacological model: in Ras-GRF1 knockout mice, THC exposure did not induce any alteration in CB1 receptor functionality, whereas in SL327-pretreated mice, CB1 receptor downregulation was observed without significant desensitization. Matching the present data with our earlier article, we can suggest that GRK2 in this cerebral area plays a role in receptor desensitization but not in downregulation. Literature for both in vitro *(20)* and in vivo systems *(10)* has reported that for the CB1 receptor, the processes of desensitization and internalization can be dissociated: the findings of the present work represent more evidence supporting this view.

To summarize, the main role of THC-induced ERK activation in the striatum and cerebellum is the modulation of trafficking proteins involved in CB1 receptor activity, whereas in the hippocampus and—although to a lesser extent—in the prefrontal cortex, other kinases may account for this phenomenon.

To define the role of THC-induced ERK activation in these latter cerebral regions, the nuclear levels of the transcription factors closely related to ERK—namely, pCREB and Fos B—were monitored. Altering gene expression is one of the main roles of the ERK cascade that has been involved in the modulation of synaptic plasticity. Chronic THC treatment significantly increased nuclear amounts of pCREB and Fos B both in the hippocampus and prefrontal cortex of wild-type mice, whereas this event did not occur in either the genetic or pharmacological model. This finding suggests that the ERK pathway contributes to initiation of a gene expression program in response to chronic cannabinoids by activating CREB and Fos B. A major interest was identifying target genes through which these transcription factors could modulate cellular activity (i.e., synaptic plasticity) in these cerebral areas. One relevant downstream protein modulated by CREB-dependent transcription is the neurotrophin BDNF *(27)*, which has been a focus of intense interest for its key role in activity-dependent neuronal plasticity in the adult brain. Synaptic activity upregulates BDNF gene expression and stimulates BDNF protein release into synapses, enhancing synaptic transmission and neuronal excitability—particularly in the hippocampus *(28–33)*. We found that chronic THC treatment significantly increased the level of BDNF in the hippocampus, and this increase was ERK-dependent because it did not occur in either Ras-GRF1 knockout mice or SL327-pretreated animals. Because the hippocampus is involved in declarative memory *(34)* and it has been related to the learning of affective states in relationship to drug intake, we suggest that chronic cannabinoid exposure affects the cellular mechanisms involved in triggering synaptic plasticity underlying learning and memory. To further confirm the role of ERK-dependent mechanisms in these phenomena, future experi-

ments should test the cognitive performance of wild-type, knockout and SL327-pretreated mice during chronic THC exposure.

Finally, no significant alterations were observed in prefrontal cortex levels of BDNF following chronic THC exposure, suggesting that downstream proteins could be different, depending on the brain region considered. However, the observed increase in pCREB and Fos B may contribute, although in an as yet unclear manner, to alteration of prefrontal cortical functions related to the process of addiction, such as self-regulation and impulse control.

In a recent study, Butovsky and collegues *(35)* found that chronic THC treatment induced a small increase of BDNF protein in the medial prefrontal cortex and no changes in the hippocampus. The discrepancy between these findings and ours could lie in the different protocol used for the chronic treatment (10 mg/kg twice a day for 5 d in our work and 1.5 mg/kg daily for 7 d in theirs), suggesting that different brain areas differ in their dose–response to cannabinoids.

As a whole, our data suggest that in the striatum and cerebellum, THC-induced ERK activation could represent a key signaling event to initiation of homologous desensitization of CB1 receptor, accounting for the development of tolerance to THC-induced hypolocomotion. This view is strengthened by the key role that these areas play in controlling motor activity. In the prefrontal cortex and hippocampus, THC-induced ERK activation could lead to impaired plasticity that might be part of the molecular underpinnings inducing the complex behavioral phenotype that defines the addicted state.

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