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Delta FosB and AP-1-mediated transcription modulate cannabinoid CB₁ receptor signaling and desensitization in striatal and limbic brain regions

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Abstract

Repeated ⁹-tetrahydrocannabinol (THC) administration produces cannabinoid type 1 receptor (CB₁R) desensitization and downregulation, as well as tolerance to its in vivo pharmacological effects. However, the magnitude of CB₁R desensitization varies by brain region, with CB₁Rs in the striatum and its output nuclei undergoing less desensitization than other regions. A growing body of data indicates that regional differences in CB₁R desensitization are produced, in part, by THC-mediated induction of the stable transcription factor, FosB, and subsequent regulation of CB₁Rs. The purpose of the present study was to determine whether THC-mediated induction of FosB in the striatum inhibits CB₁R desensitization in the striatum and output nuclei. This hypothesis was tested using bitransgenic mice with inducible expression of FosB or cJun, a dominant negative inhibitor of AP-1-mediated transcription, in specific forebrain regions. Mice were treated repeatedly with escalating doses of THC or vehicle for 6.5 days, and CB₁R-mediated G-protein activation was assessed using CP55,940-stimulated [³⁵S]GTPγS autoradiography. Overexpression of FosB in striatal dopamine type 1 receptor-containing (D₁R) medium spiny neurons (MSNs) attenuated CB₁R desensitization in the substantia nigra, ventral tegmental area (VTA) and amygdala. Expression of cJun in striatal D₁R- and dopamine type 2 receptor (D₂R)-containing MSNs enhanced CB₁R desensitization in the caudate-putamen and attenuated desensitization in the hippocampus and VTA. THC-mediated in vivo pharmacological effects were then assessed in cJun-expressing mice. Tolerance to THC-mediated hypomotility was enhanced in cJun-expressing mice. These data reveal that FosB and possibly other AP-1 binding proteins regulate CB₁R signaling and adaptation in the striatum and limbic system.

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Keywords

⁹-tetrahydrocannabinol; striatum; amygdala; basal ganglia; G-protein; dopamine receptor

Introduction

⁹-Tetrahydrocannabinol (THC), the main psychoactive constituent of marijuana, elicits behavioral effects by activating cannabinoid CB₁ receptors (CB₁Rs) in the central nervous system [1, 2]. Repeated THC administration produces CB₁R desensitization, measured as a reduction in receptor-mediated G-protein or effector activity [3], which occurs concomitantly with tolerance to THC-mediated in vivo effects [4]. CB₁R desensitization varies in magnitude by brain region depending on the dose and duration of repeated cannabinoid administration. Moreover, the brain regional profile of desensitization corresponds with the development of tolerance to specific cannabinoid-mediated responses [3]. For example, CB₁R desensitization in the dorsal striatum and its output nuclei (i.e., globus pallidus, substantia nigra) is generally lower in magnitude and requires higher drug doses and/or longer treatment duration than other brain regions [3, 5, 6]. Studies using post-mortem autoradiography or in vivo imaging in brains from marijuana users compared to non-users have also revealed a smaller decrease in CB₁R levels in the caudate-putamen compared to other regions, including hippocampus [7, 8]. These findings are consistent with reports in human marijuana users that showed greater tolerance to the memory impairing effects of THC, which are hippocampal-dependent, than to motor impairment and subjective “high”, which involve striatal circuits [9]. These data suggest the potential functional and translational relevance of regional differences in CB₁R adaptation, but the regulatory mechanisms that underlie these region-specific effects are not known.

We have proposed that regional differences in the interaction of CB₁Rs with specific signaling and regulatory proteins contribute to region-specific differences in CB₁R adaptation [3, 10, 11] and recently suggested that the induction of transcription factors following repeated THC administration plays a modulatory role in CB₁R desensitization [12]. This idea was based, in part, on the demonstration that an inverse regional relationship exists between THC-mediated CB₁R desensitization and induction of FosB [13]. For example, repeated THC treatment induces FosB expression in the striatum, a region that generally shows lower CB₁R desensitization. FosB belongs to the Fos family of transcription factors, which dimerize with Jun proteins to produce an AP-1 complex that regulates the transcription of target genes [14, 15]. FosB, a truncated splice variant of FosB, is stable and accumulates with repeated drug administration [16]. Inducible transgenic overexpression of FosB in dopamine type 1 receptor (D₁R) positive striatal medium spiny neurons (MSNs) showed enhanced rewarding effects of drugs of abuse and natural rewards [17, 18]. In contrast, expression of cJun, a dominant negative inhibitor of FosB-mediated transcription, in both D₁R and dopamine type 2 receptor (D₂R) positive MSNs reduced cocaine- and morphine- induced condition place preference [19, 20]. FosB produces functional effects by regulating the expression of target genes that include certain receptors and signaling proteins [21], which can affect receptor-mediated signaling. For example, inducible transgenic overexpression of FosB in the nucleus accumbens enhanced mu

opioid, but not CB₁, receptor-mediated G-protein activity and inhibition of adenylyl cyclase [22]. Although FosB might not play a pivotal role in CB₁R signaling acutely, it might modulate CB₁R desensitization. The current study tested the hypothesis that repeated THC administration to transgenic mice that inducibly overexpress FosB or its transcriptional inhibitor cJun in the forebrain will display a reduction in CB₁R desensitization, as assessed by CP55,940-stimulated [³⁵S]GTPγS binding. Additionally, tolerance to common pharmacological effects (hypomotility, antinociception, catalepsy, and hypothermia) of THC was quantified in transgenic and wild type mice treated repeatedly with vehicle or THC.

Materials and Methods

Materials

THC and [(–)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol] (CP55,940) and SR141716A were provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). [³⁵S]Guanosine 5'-(gamma-thio)triphosphate (GTPγS; 1250 Ci/mmol), [³H]SR141716A (54 Ci/mmol) and Kodak Biomax MR film were purchased from PerkinElmer Life Sciences (Boston, MA). Bovine serum albumin (BSA), guanosine diphosphate (GDP) and doxycycline were purchased from Sigma-Aldrich (St. Louis, MO). ScintiSafe Econo 1 scintillation fluid was obtained from Research Products International (Mount Prospect, IL). Whatman glass fiber filters were purchased from Brandel (Gaithersburg, MD). All other reagent grade chemicals were obtained from Sigma Chemical Co. or Fisher Scientific.

Subjects and Drug Treatments

Subjects were male, bitransgenic *NSE-tTA* × *TetOp-FosB* mice and *NSE-tTA* × *TetOp-FLAG-c-Jun* mice with brain-region specific, tetracycline-regulated inducible expression of either FosB or cJun, respectively [19, 23]. FosB or cJun expression is controlled by adding doxycycline to the drinking water, which prevents FosB/ cJun expression. Therefore, omission of doxycycline from the drinking water allows FosB/ cJun to be expressed. In mice that overexpress FosB, expression is found in D₁R-positive MSNs in the caudate-putamen and nucleus accumbens, deep layers of cerebral cortex and hippocampus [23]. In mice that overexpress cJun, expression occurs in both D₁R and D₂R positive MSNs in the caudate-putamen and nucleus accumbens, parietal cortex and hippocampus [19]. cJun is a dominant negative functional inhibitor of AP-1-mediated transcription, thus this model provides a strategy to block the effects of THC-induced FosB expression. Mice were housed four to six per cage and maintained on a 12-hr light/dark cycle in a temperature-controlled environment (20–22°C) with food and water available ad libitum. Mice were maintained on drinking water that contained doxycycline (100 µg/ml) throughout gestation and were either taken off doxycycline for 8 weeks prior to experiments to induce expression of FosB or cJun (FosB-ON, cJun-ON) or maintained on doxycycline (control; FosB-OFF, cJun-OFF). After 8 weeks with/without doxycycline, mice were injected subcutaneously twice daily (08:00 and 16:00) for 6 days with vehicle (1:1:18 solution of ethanol, emulphor and saline) or THC doses that increased every 2 days (10-30-60 mg/kg, injection) [5]. On day 7, mice for autoradiographic studies received only the morning injection of THC or vehicle, and 24 hours later brains were collected for

CP55,940-stimulated [^{35}S]GTP γ S binding. This THC treatment regimen was employed because it produces CB $_1$ R desensitization throughout the brain, including the striatum, and therefore should reveal whether FosB expression alters CB $_1$ R desensitization. All experiments were performed with the approval of the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health guide for the care and use of Laboratory animals 8th edition.

Agonist-stimulated [^{35}S]GTP γ S Autoradiography

Assays were conducted as previously published [10, 24]. Coronal brain sections (20 μm) were cut on a cryostat maintained at -20°C , thaw-mounted onto gelatin-coated slides and stored desiccated at 4°C overnight. Sections were collected at levels that included 1) prefrontal cortex, 2) nucleus accumbens, 3) caudate-putamen, 4) globus pallidus, 5) hippocampus and amygdala (including central, basolateral and basomedial nuclei), 6) VTA, 7) substantia nigra, and 8) cerebellum. Slides were stored desiccated at -80°C until use. On the day of the assay, slides were brought to room temperature, rinsed in 50 mM Tris-HCl buffer (pH 7.4) with 3 mM MgCl_2 , 0.2 mM EGTA and 100 mM NaCl (TME Buffer) for 10 min at 25°C . Slides were transferred to TME Buffer + 0.5% BSA with 2 mM GDP and 10 mU/ml adenosine deaminase for 15 min at 25°C . Slides were then incubated in TME Buffer + 0.5% BSA containing 0.04 nM [^{35}S]GTP γ S, 2 mM GDP, 10 mU/ml adenosine deaminase and 3 μM CP55,940 or vehicle (ethanol) for 2 hours at 25°C . CP55,940 was selected because it does not stimulate [^{35}S]GTP γ S binding in brain sections from CB $_1$ R knockout mice [25], thereby minimizing the possibility of off target activity. The maximally effective concentration of CP55,940 was previously determined in assays of cerebellar sections and homogenates [25]. After final incubation, slides were rinsed twice in 50 mM Tris buffer (pH 7.4) at 4°C , and then in deionized water. Slides were dried and exposed to Kodak Biomax MR film with [^{14}C] microscaler for 18 hrs. Films were digitized at 8-bits per pixel with a Sony XC-77 video camera. Brain regions of interest (ROIs) were determined using The Mouse Brain Atlas [26]. Images were analyzed using NIH ImageJ software, and resulting values are expressed as nanocuries (nCi) of [^{35}S] per gram of tissue as previously described [27].

In vivo Assessment

Mice ($n = 8/\text{group}$) were evaluated 24 hours after the last THC injection to determine whether overexpression of cJun affected THC-induced responses after repeated vehicle or THC treatment. Mice were challenged with a single dose of THC that was determined based on dose-response data from our previous studies [28, 29]. Baseline measures were first assessed in the absence of THC, and then mice received intraperitoneal (i.p.) injection of 100 mg/kg THC and were assessed for locomotor activity, immobility, rectal temperature and warm water tail withdrawal [28, 29]. To assess locomotor activity, each mouse was placed in a clear Plexiglas box ($42.7 \times 21.0 \times 20.4$ cm) for a 5-minute assessment period and Anymaze software (Stoelting, Wood Dale, Illinois) was used to determine the amount of time spent immobile [30]. Mice were tested in separate chambers for baseline and THC trials to avoid habituation. Catalepsy was determined in the bar test, antinociception was evaluated in the warm water tail immersion test at 52.0°C , and body temperature was measured by inserting a thermocouple probe 2.0 cm into the rectum as we have published

[28]. Baseline measures were taken, and then mice were injected with THC and tested 20 minutes later for locomotor activity. Catalepsy, antinociception and hypothermia were tested 3 hours after THC injection because initial studies determined that maximal effects were produced at this time point (data not shown).

[³H]SR141716A Binding

Brains from mice used for in vivo testing were collected 24 hours after evaluation and stored at – 80°C. Regions of interest (caudate-putamen, hippocampus, amygdala) were dissected and membranes were prepared as described previously [6]. 5–12 µg membrane protein was incubated with 0.05–5 nM [³H]SR141716A in TME buffer with 0.5% bovine serum albumin (BSA) in the presence and absence of 5 µM unlabeled SR141716A to determine non-specific and specific binding, respectively. The assay was incubated for 90 minutes at 30°C and terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that were pre-soaked in Tris buffer containing 5 g/L BSA (Tris-BSA), followed by five washes with cold Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency in ScintiSafe Econo 1 scintillation fluid after a 12-hour delay.

Data Analysis

Net agonist-stimulated [³⁵S]GTPγS binding was calculated by subtracting basal (without agonist) binding from agonist-stimulated binding. Values were obtained in triplicate sections collected from ten brains per group and averaged for statistical analysis. Data from all experiments were analyzed with Prism® version 5 (GraphPad Software, San Diego, CA). Two-way ANOVA with Bonferroni post-hoc test was used to determine significant differences in net agonist-stimulated [³⁵S]GTPγS binding. Desensitization was calculated as (net-stimulated [³⁵S]GTPγS binding in THC-treated mice / net-stimulated [³⁵S]GTPγS binding in vehicle-treated mice) × 100, and Student's t-tests were used based on planned comparisons by region. For [³H]SR141716A binding, B_{max} and K_D values were calculated by iterative fitting of the saturation curves to the Langmuir equation [$B = (B_{\max} \times \text{ligand concentration}) / (K_D + \text{ligand concentration})$]. For in vivo studies, data were analyzed by two-way ANOVA with Bonferroni post-hoc test. Hypothermia is presented as the change in temperature from baseline measures and tail withdrawal is presented as percentage of maximum possible effect ($[(\text{test latency} - \text{baseline latency}) / 10 \text{ seconds} - \text{baseline latency}] \times 100$). Significance was determined with $p < 0.05$ and all results are presented as mean ± SEM.

Results

CB₁R desensitization is attenuated in the ventral midbrain and amygdala of mice that overexpress FosB

The effect of repeated THC administration on CP55,940-stimulated [³⁵S]GTPγS binding in the brains of FosB-ON and FosB-OFF mice is shown in representative sections in Figure 1. Repeated THC appeared to reduce CP55,940-stimulated [³⁵S]GTPγS binding in multiple brain regions, with regional variation in the magnitude of the effect. Densitometric analysis was then performed to quantify [³⁵S]GTPγS binding in each region. Basal levels of

[³⁵S]GTPγS binding did not differ between any group of FosB-ON and FosB-OFF mice in any region examined (data not shown). Net CP55,940-stimulated [³⁵S]GTPγS binding was then compared between vehicle-treated FosB-ON and FosB-OFF mice to determine whether FosB expression altered cannabinoid-mediated G-protein activity in drug-naïve mice (Figure 2, Table 1). Agonist-stimulated [³⁵S]GTPγS binding in the amygdala was 14% lower ($p < 0.01$) in FosB-ON mice compared to FosB-OFF mice. No differences in CP55,940-stimulated [³⁵S]GTPγS binding were found between vehicle-treated FosB-ON and FosB-OFF mice in any other region examined. The effects of repeated THC administration on CP55,940-stimulated [³⁵S]GTPγS binding were then compared between FosB-ON and FosB-OFF mice (Figure 2, Table 1). CP55,940-stimulated [³⁵S]GTPγS binding was significantly lower in repeated THC- compared to vehicle-treated brains from both FosB-OFF and FosB-ON mice in almost all regions examined. The exception was the VTA, where there was no significant difference in CP55,940-stimulated [³⁵S]GTPγS binding between vehicle- and THC-treated FosB-ON mice. In contrast, FosB-OFF mice treated repeatedly with THC exhibited a significant reduction of in CP55,940-stimulated [³⁵S]GTPγS binding. Although both FosB-OFF and FosB-ON mice showed significant THC-induced desensitization of agonist-stimulated [³⁵S]GTPγS binding in the substantia nigra, FosB overexpression decreased the magnitude and significance level of this effect. In FosB-OFF mice, repeated THC reduced CP55,940-stimulated [³⁵S]GTPγS binding to $56 \pm 3\%$ of the response obtained in vehicle-treated mice. In FosB-ON mice, however, repeated THC only reduced stimulation by CP55,940 to $78 \pm 4\%$ of that obtained in vehicle-treated mice. Likewise, in amygdala, FosB overexpression decreased THC-induced desensitization of CP55,940-stimulated [³⁵S]GTPγS binding, from $35 \pm 3\%$ of vehicle control in FosB-OFF mice to $45 \pm 2\%$ of vehicle control in FosB-ON mice. Thus, inducible transgenic overexpression of FosB attenuated desensitization of CB₁R-mediated G-protein activation by repeated THC in VTA, substantia nigra and amygdala, and reduced CB₁R-mediated G-protein activation in the amygdala of vehicle-treated mice.

CB₁R desensitization is enhanced in the caudate-putamen and reduced in the hippocampus and VTA of cJun-ON mice

Studies were also conducted to determine whether the expression of cJun, a dominant negative inhibitor of AP-1-mediated transcription, would alter CB₁R-mediated G-protein activity or desensitization. cJun-ON and cJun-OFF mice received the same repeated THC treatment as the FosB overexpressing mice in order to directly compare results. CP55,940-stimulated [³⁵S]GTPγS binding is shown in representative brain sections in Figure 3 and densitometric analysis was conducted in the same regions described above (Table 2). Basal levels of [³⁵S]GTPγS binding did not differ between any group of cJun-ON and cJun-OFF mice in any region examined (data not shown). Analysis of brains from vehicle-treated mice revealed that CP55,940-stimulated [³⁵S]GTPγS binding in the amygdala was 29% greater ($p < 0.01$) in cJun-ON compared to cJun-OFF mice ($p < 0.01$, Figure 4, Table 2). CP55,940-stimulated [³⁵S]GTPγS binding did not differ between cJun-ON and cJun-OFF vehicle-treated mice in any other region examined. Repeated THC treatment in cJun-OFF mice significantly reduced CP55,940-stimulated [³⁵S]GTPγS binding compared to vehicle-treatment in all regions examined, except for the caudate-putamen (Figure 4, Table 2). In contrast, THC-treated cJun-ON mice displayed a significant decrease in CP55,940-

stimulated [35 S]GTP γ S binding, compared with vehicle-treated cJun-ON mice, in all brain regions assessed, including the caudate-putamen (Figure 4, Table 2). In cJun-OFF mice, repeated THC reduced CP55,940-stimulated [35 S]GTP γ S binding in caudate-putamen to $62 \pm 13\%$ of the response obtained in vehicle-treated mice. In cJun-ON mice, however, repeated THC treatment reduced stimulation by CP55,940 to $39 \pm 4\%$ of that obtained in vehicle-treated mice, suggesting that cJun expression enhanced CB $_1$ R desensitization. In the hippocampus of cJun-OFF mice, CP55,940-stimulated [35 S]GTP γ S binding was decreased by repeated THC treatment to $18 \pm 4\%$ of that observed in vehicle-treated mice, whereas in THC- cJun-ON mice, CP55,940 stimulation was $37 \pm 6\%$ of that seen in vehicle-treated mice. Similarly, less CB $_1$ R desensitization was also found in the VTA of THC-treated cJun-ON compared to cJun-OFF mice ($36 \pm 4\%$ of vehicle in cJun-ON vehicle-treated mice vs. $24 \pm 3\%$ of vehicle in cJun-OFF vehicle-treated mice). Thus, inducible transgenic expression of cJun increased CB $_1$ R desensitization in the caudate-putamen, decreased desensitization in hippocampus and VTA and enhanced CB $_1$ R-stimulated G-protein activation in the amygdala.

Tolerance to THC-mediated locomotor suppression is enhanced in cJun-ON mice following repeated THC administration

Because desensitization was enhanced in the caudate-putamen of cJun-ON mice, we assessed whether blocking AP-1-mediated transcription would affect tolerance to THC-mediated motor effects (i.e., locomotor suppression and catalepsy), as well as other common pharmacological effects of this drug (i.e., hypothermia and analgesia). Baseline measures were first determined prior to repeated injections. Baseline measures for locomotor immobility ranged from 14.0 ± 3.5 to 20.1 ± 4.6 sec and did not significantly differ between cJun-ON and cJun-OFF in drug-naïve mice. The baseline rectal temperatures of cJun-ON and cJun-OFF mice ranged from $38.0 \pm 0.2^\circ\text{C}$ to $38.9 \pm 0.1^\circ\text{C}$ and did not significantly differ from each other. Baseline tail flick latencies ranged from 4.0 ± 0.3 s to 4.4 ± 0.4 s and were not significantly different among groups. Catalepsy was not observed for any group at baseline. THC injection produced hypothermia, antinociception, catalepsy and locomotor suppression in repeated vehicle-treated cJun-OFF and cJun-ON mice (Figure 5). There was a significant interaction ($F_{1, 30} = 8.69$, $p < 0.01$) between repeated THC treatment and cJun expression for THC-mediated locomotor suppression. Bonferroni post-hoc analysis showed that repeated THC-treated cJun-ON mice exhibited significantly less locomotor suppression compared to repeated THC-treated cJun-OFF mice ($p < 0.05$), suggesting enhanced tolerance. Repeated THC administration significantly affected hypothermia ($F_{1, 32} = 80.98$, $p < 0.001$), but no main effect of cJun expression or an interaction between treatment and cJun status was found. Similarly, there was a significant main effect of repeated THC treatment for antinociception ($F_{1, 32} = 78.12$, $p < 0.001$), but no main effect of cJun expression or interaction. For catalepsy, significant main effects of both repeated THC treatment ($F_{1, 32} = 37.35$, $p < 0.001$) and cJun expression ($F_{1, 32} = 20.98$, $p < 0.001$) were found, indicating that repeated THC attenuated, whereas cJun expression enhanced, acute THC-induced catalepsy. These results indicate that greater tolerance to THC-mediated locomotor suppression developed in cJun-OFF compared to cJun-ON mice, whereas tolerance to catalepsy, hypothermia and antinociception did not significantly differ between mice with and without cJun expression.

CB₁R binding and downregulation do not differ between cJun-ON and cJun-OFF mice

[³H]SR141716A saturation binding was conducted in the caudate-putamen, hippocampus and amygdala of cJun-ON and cJun-OFF mice to determine whether G-protein activation results might be due to differences in CB₁R expression produced by blocking AP-1 mediated transcription (Table 3). Results in vehicle-treated mice showed that neither the B_{max} nor K_D values for [³H]SR141716A binding differed between cJun-ON and cJun-OFF mice in any region examined, as determined by a lack of main effect of transgene expression by two-way ANOVA. Therefore, genotype-specific differences in CB₁R signaling or THC-mediated in vivo effects do not appear to result from transgene-induced alterations in CB₁R expression. The effect of THC on CB₁R expression in cJun-ON and cJun-OFF mice was then determined. In caudate-putamen, there was no main effect of repeated treatment on [³H]SR141716A B_{max} values ($F_{1,18} = 3.627$, $p = 0.073$), nor was there an interaction with cJun expression ($F_{1,18} = 0.377$, $p = 0.547$). Similar results were seen in amygdala, where there was no main effect of repeated treatment on B_{max} values ($F_{1,18} = 0.068$, $p = 0.796$), nor an interaction with cJun expression ($F_{1,18} = 1.948$, $p = 0.18$). In contrast, repeated THC downregulated [³H]SR141716A B_{max} values in hippocampus ($F_{1,20} = 12.71$, $p = 0.002$). However, there was no interaction between cJun expression and repeated treatment ($F_{1,20} = 1.276$, $p = 0.272$). [³H]SR141716A K_D values were not significantly affected by repeated THC treatment, nor was there an interaction with cJun expression, in any region examined. These results indicate that CB₁Rs are downregulated in the hippocampus by repeated THC treatment regardless of cJun expression, and that expression of the transgene itself did not influence CB₁R expression levels..

Discussion

This study investigated whether FosB expression modulates CB₁R-mediated G-protein signaling or desensitization of this response after repeated THC administration. Results in drug-naïve mice revealed that FosB overexpression reduced, whereas cJun expression increased, CB₁R-mediated G-protein activity in the amygdala. No differences in CB₁R signaling were found in other regions. We proposed that induction of FosB in the striatum would inhibit THC-induced CB₁R desensitization in the striatum and/or its output nuclei based in part on the inverse regional relationship previously identified between THC-induced CB₁R desensitization and FosB expression [13]. The current study showed that overexpression of FosB in D₁R-positive MSNs attenuated THC-induced CB₁R desensitization in the amygdala, substantia nigra and VTA in mice treated with a THC regimen that produces CB₁R desensitization throughout the brain. cJun overexpression was then used to functionally inhibit transcription by THC-induced AP-1 binding partners, including FosB. Previous studies showed that THC treatment induces FosB expression in the striatum [13], thus the transcriptional effects of FosB would be blocked in mice expressing cJun. Results showed that expression of cJun enhanced CB₁R desensitization in the caudate-putamen, but attenuated desensitization in the hippocampus and VTA. Subsequent studies showed that tolerance to THC-mediated hypomotility was enhanced in cJun-expressing mice, consistent with G-protein activation results in the caudate-putamen. Because CB₁Rs have an AP-1 site in their promoter region [31], CB₁R binding was assessed in cJun control and overexpressing mice. However, no differences in CB₁R expression

were found between genotypes. These findings build on our previous report [13], which used a correlative approach to determine the anatomical relationship between expression of

FosB and CB₁R desensitization. The current study employed genetic models that suggest a cause and effect relationship between expression of FosB and AP-1 binding proteins and the regulation of CB₁R-mediated G-protein signaling and desensitization. Taken together, these results support the idea that FosB inhibits CB₁R desensitization in the striatum and a subset of its projection regions, the substantia nigra and VTA, and that AP-1 binding proteins regulate CB₁R signaling and adaptation in the limbic system.

The bitransgenic mouse lines used in these studies inducibly overexpress FosB or cJun in the caudate-putamen, nucleus accumbens, cerebral cortex and hippocampus [19, 23]. Both

FosB and cJun are inducibly expressed in D₁R/dynorphin-expressing MSNs of the caudate-putamen and nucleus accumbens and cJun is also expressed in D₂R/enkephalin-expressing MSNs. Striatal MSNs have been distinguished based on expression of D₁Rs or D₂Rs, which comprise the direct and indirect pathways, respectively [32, 33]. Recent studies have confirmed that the D₁R containing direct pathway from the striatum to the midbrain facilitates motor activity and reward, whereas the D₂R containing indirect pathway to the pallidum inhibits these processes [34]. The mouse lines used in this study have previously been used to demonstrate that expression of FosB in D₁R-positive MSNs enhances both drug and natural rewards [17, 18]. Moreover, recent studies showed that administration of drugs of abuse, including THC, to fluorescent reporter BAC transgenic mice induced FosB in D₁R positive MSNs [35]. In fact, THC administration enhanced FosB expression in D₁R, but not D₂R, positive neurons in the dorsal striatum and nucleus accumbens shell and core. In the current study, transgenic expression of FosB or cJun did not affect CB₁R signaling in the caudate-putamen or nucleus accumbens of drug naïve mice, which we previously reported in membrane homogenates prepared from the nucleus accumbens of these mice [22]. However, overexpression of FosB attenuated CB₁R desensitization in the substantia nigra and VTA, which are innervated by D₁R-positive MSNs of the caudate-putamen and nucleus accumbens, respectively. CB₁Rs have been localized to D₁R positive MSNs and their projections to the midbrain [36], thus altered regulation of CB₁R signaling by THC-induced FosB has the potential to modulate motor and motivational effects produced by THC as well as other drugs.

These studies employed a THC dosing regimen that we have previously shown produces CB₁R desensitization in the striatum [5], so that either reversal or augmentation of desensitization by transgene expression would be revealed. This dosing regimen also produces THC levels that are consistent with reported values in human marijuana users. While the THC blood levels were not quantified in the present study, we previously reported that 56 mg/kg THC administered intraperitoneally in mice resulted in a THC blood concentration of 1054 ng/ml at 30 minutes [28]. This concentration translates to the human equivalent concentration of 85 ng/ml THC based on differences of surface area between the species [37], and is in alignment with the 51 ng/ml THC blood levels found in humans 30 minutes after smoking a marijuana cigarette containing 3.55% THC [38].

Expression of cJun, a functional inhibitor of AP-1-mediated transcription, enhanced CB₁R desensitization in the caudate-putamen, as predicted. However, FosB overexpression did

not reduce desensitization in this region. It is possible that results in the FosB overexpressing mice reflect the restricted expression of FosB to D₁R-positive MSNs. CB₁Rs in the striatum are expressed by both D₁R- and D₂R-containing MSNs, as well as on glutamatergic afferent projections [39, 40]. Accordingly, analyzing the combined CB₁R populations in this region might have masked the effects of FosB overexpression. It is also possible that the doses of THC administered in this study were sufficient to overcome the inhibitory effect of FosB on CB₁R desensitization or that the level of FosB induced by repeated THC is already maximally effective. Thus, transgenic overexpression of FosB might not be capable of further reducing CB₁R desensitization.

The functional consequence of FosB-mediated modulation of CB₁R desensitization was suggested by results showing that transgenic expression of cJun enhanced tolerance to the locomotor suppressive effects of THC. This result is consistent with the finding that cJun expression enhanced CB₁R desensitization in the caudate-putamen. Interestingly, cJun expression modestly, but significantly, enhanced THC-induced catalepsy. As noted above, cJun is expressed in both D₁R and D₂R expressing MSNs in the striatum and inhibits AP-1 mediated transcription by FosB and other Fos family members, so it is unclear which actions of cJun are responsible for this effect. Reports in the literature regarding MSN populations that could mediate CB₁R-induced catalepsy are conflicting. One study found that targeted deletion of CB₁Rs in D₁R-expressing neurons attenuated cannabinoid-induced catalepsy [41], whereas another study showed evidence for involvement of D₂R-expressing neurons in CB₁R-mediated catalepsy [42]. Because repeated THC induces FosB mainly in D₁R-expressing neurons, one interpretation of the in vivo results in the cJun model is that enhanced CB₁R desensitization and tolerance to locomotor suppression was mediated by antagonism of FosB by cJun expression in D₁R-expressing MSNs. In contrast, enhancement of catalepsy might have been due to antagonism of undetermined Fos family members in D₂R-expressing MSNs. In any case, there were no significant effects of cJun expression on hypothermia or thermal antinociception, indicating selective effects of AP-1-mediated transcription on acute and chronic effects of THC on neurotransmission associated with motor function.

FosB and cJun expression also modulated CB₁R signaling in the amygdala. This finding is surprising because neither FosB nor cJun is inducibly expressed in the amygdala of these transgenic mice [19, 23] and basal levels of FosB are normally low in this region [43]. Overexpression of FosB attenuated CB₁R-mediated G-protein activation in the amygdala, whereas expression of cJun enhanced activity, in drug-naïve mice. Overexpression of FosB also attenuated THC-induced CB₁R desensitization in the amygdala, further supporting a role for this transcription factor in CB₁R signaling in this region. CB₁Rs are expressed on both GABAergic interneurons and glutamatergic afferents in the amygdala [44, 45]. Selective deletion of CB₁Rs from calcium/calmodulin-dependent protein kinase II (CaMKII α) expressing principal neurons abolishes the ability of WIN55212-2, a high efficacy cannabinoid receptor agonist, to reduce evoked excitatory postsynaptic responses [44]. Further investigation revealed that CB₁Rs were expressed on glutamatergic presynaptic axon terminals. Thus, it is likely that the effects of FosB and cJun on CB₁R-mediated G-protein activity in the amygdala were due to alterations in CB₁R signaling on glutamatergic afferents from cortical principal neurons in which FosB

or cJun were overexpressed. The finding that FosB modulates CB₁R signaling and desensitization in the amygdala suggests this signaling pathway could alter cannabinoid effects on motivation and anxiety.

It is surprising that CB₁R desensitization was attenuated in the hippocampus of cJun expressing mice, because FosB is not induced by repeated THC administration in this region [13] and desensitization was not altered in the hippocampi of FosB overexpressing mice. A likely explanation for this finding is that cJun inhibits transcriptional regulation by other Fos family members [19]. Both c-Fos and FosB are likely candidates because they are induced in the nucleus accumbens and hippocampus by THC treatment [46, 47, 48]. In contrast, expression of cJun did not significantly affect CB₁R downregulation in the hippocampus. The mechanism(s) underlying CB₁R desensitization in this region is not clear, but these results suggest a role for Fos family transcription factors and target genes that regulate CB₁R signaling.

The present study supports a role for FosB in modulating CB₁R signaling and desensitization in the forebrain. The findings support the hypothesis that FosB, which can be induced by THC as well as other stimuli, inhibits CB₁R desensitization in the striatal system. Results also revealed that FosB and cJun modulate CB₁R signaling in the amygdala of both drug-naïve and THC-treated mice. Finally, a role for Fos family proteins in regulating CB₁R desensitization in the hippocampus was indicated in cJun expressing mice. These regions contribute to the neurocircuitry that mediates the transition from drug use to addiction [49]. The transcriptional targets of FosB/Fos that might regulate CB₁R desensitization are not known. Diverse genes are regulated by FosB in the nucleus accumbens and include receptors, signaling proteins and cytoskeletal elements [21]. For example, overexpression of FosB upregulates Gα_o and several kinases (e.g. CaMKIIα, protein kinase Cβ) in the nucleus accumbens [21]. CB₁Rs are desensitized by the GRK-βarrestin pathway [10, 50], which might be directly or indirectly regulated by AP-1-mediated transcription. The region-specific effects of overexpression of FosB or cJun on CB₁R signaling and desensitization likely result, in part, from regional differences in co-localization of CB₁R with gene products whose expression is regulated by AP-1 transcriptional control. The current results provide novel mechanism(s) by which THC-mediated FosB induction and CB₁R desensitization can interact to regulate CB₁R activity in response to repeated THC administration.

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Abbreviations

AMYG	amygdala
BSA	bovine serum albumin
CaMKIIα	calcium/ calmodulin-dependent protein kinase II

CB₁R	cannabinoid type 1 receptor
CBLM	cerebellum
CP55	940, (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol
CPU	caudate-putamen
D₁R	dopamine type 1 receptor
D₂R	dopamine type 2 receptor
ERK	extracellular signal-regulated kinase
GP	globus pallidus
GDP	guanosine diphosphate
GTPγS	guanosine 5''-(gamma-thio)triphosphate
HIP	hippocampus
MSN	medium spiny neuron
NAC	nucleus accumbens
PFC	prefrontal cortex
SN	substantia nigra
THC	⁹ -tetrahydrocannabinol
VTA	ventral tegmental area

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Figure 1.

Representative autoradiograms showing CP55,940-stimulated [35 S]GTP γ S binding in FosB-ON and FosB-OFF mice following repeated vehicle or THC (10-30-60 mg/kg, b.i.d., 6.5 days) treatment. CPU, caudate-putamen; GP, globus pallidus; HIP, hippocampus; AMYG, amygdala; SN, substantia nigra

[³⁵S]GTPγS binding in ΔFosB ON and OFF mice

Figure 2.

Net CP55,940-stimulated [³⁵S]GTPγS binding in brain regions of vehicle- and THC-treated

FosB-ON and FosB-OFF mice expressed as percent of net-stimulated binding in vehicle-treated FosB-OFF mice. Vehicle-treated FosB-ON mice exhibited significantly less net-stimulated [³⁵S]GTPγS binding in the amygdala compared to FosB-OFF mice. Net-stimulated [³⁵S]GTPγS binding was significantly decreased in all brain regions of FosB-ON and FosB-OFF mice after THC treatment, with the exception of the VTA of FosB-ON mice. Data are presented as means ± SEM (n = 8–10 mice per group) * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to vehicle-treated FosB-OFF mice and # p < 0.05, ## p < 0.01, ### p < 0.001 as compared to vehicle-treated FosB-ON mice following two-way ANOVA and Bonferroni post-hoc tests.

Figure 3.

Representative autoradiograms showing CP55,940-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in $\Delta\text{cJun-ON}$ and $\Delta\text{cJun-OFF}$ mice following repeated vehicle or THC (10-30-60 mg/kg, b.i.d., 6.5 days) treatment. CPU, caudate-putamen; GP, globus pallidus; HIP, hippocampus; AMYG, amygdala; SN, substantia nigra

[³⁵S]GTPγS binding in ΔcJun ON and OFF mice **Figure 4.**

Net CP55,940-stimulated [³⁵S]GTPγS binding in brain regions of vehicle- and THC-treated cJun-ON and cJun-OFF mice expressed as percent of net-stimulated binding in vehicle-treated cJun-OFF mice. Vehicle-treated cJun-ON mice exhibited significantly greater net-stimulated [³⁵S]GTPγS binding in the amygdala compared to cJun-OFF mice. Net-stimulated [³⁵S]GTPγS binding was significantly decreased in all brain regions of THC-treated cJun-ON and cJun-OFF mice, with the exception of the caudate-putamen of cJun-OFF mice. Data are presented as means ± SEM (n = 8–10 mice per group) ** p < 0.01 and *** p < 0.001 as compared to vehicle-treated cJun-OFF mice and ## p < 0.01 and ### p < 0.001 as compared to vehicle-treated cJun-ON mice following two-way ANOVA and Bonferroni post-hoc tests.

Figure 5.

THC-mediated hypothermia (A), antinociception (B), catalepsy (C) and locomotor suppression (D) in Δ cJun-ON and Δ cJun-OFF mice following repeated vehicle or THC treatment. THC-treated Δ cJun-ON mice had significantly less locomotor suppression compared to both vehicle-treated Δ cJun-ON mice and THC-treated Δ cJun-OFF mice. Data are presented as percent of respective vehicle with mean $\pm$ SEM ($n = 8-10$ mice per group). ** $p < 0.01$, *** $p < 0.001$ as compared to vehicle-treated Δ cJun-OFF mice. # $p < 0.05$ ###, $p < 0.001$ compared to vehicle-treated Δ cJun-ON mice. ^, $p < 0.05$ compared to THC-treated control mice by two-way ANOVA following Bonferroni post-hoc tests.

TABLE 1

Net CP55,940-stimulated [35 S]GTP γ S binding in brain sections from FosB-ON and FosB-OFF mice following repeated vehicle or THC treatment.

Brain Region	Net CP55,940-stimulated [35 S]GTP γ S binding (nCi/g) $\pm$ SEM			
	FosB-OFF Vehicle	FosB-OFF THC	FosB-ON Vehicle	FosB-ON THC
Prefrontal Cortex	446 $\pm$ 21	246 $\pm$ 15***	456 $\pm$ 17	229 $\pm$ 11###
Nucleus Accumbens	403 $\pm$ 41	198 $\pm$ 37***	443 $\pm$ 33	230 $\pm$ 27###
Caudate-Putamen	205 $\pm$ 16	90 $\pm$ 13***	225 $\pm$ 10	102 $\pm$ 16###
Globus Pallidus	613 $\pm$ 54	437 $\pm$ 53*	649 $\pm$ 49	444 $\pm$ 48#
Hippocampus	273 $\pm$ 17	65 $\pm$ 7***	247 $\pm$ 17	66 $\pm$ 11###
Amygdala	393 $\pm$ 16	138 $\pm$ 11***	339 $\pm$ 12**	153 $\pm$ 7###
VTA	118 $\pm$ 13	76 $\pm$ 7*	118 $\pm$ 15	82 $\pm$ 5
Substantia Nigra	608 $\pm$ 48	339 $\pm$ 21***	558 $\pm$ 40	436 $\pm$ 24#
Cerebellum	293 $\pm$ 14	150 $\pm$ 19***	293 $\pm$ 36	143 $\pm$ 14###

Brain sections were assayed as described in Methods and autoradiograms were analyzed using densitometry. Results are expressed as net CP55,940-stimulated [35 S]GTP γ S binding (nCi/g) $\pm$ SEM, one-way ANOVA, Bonferroni post-hoc tests.

* p < 0.05,

** p < 0.01, p < 0.001 vs. FosB-OFF vehicle.

p < 0.05,

p < 0.001 vs. FosB-ON vehicle. (n = 8–10 per group)

TABLE 2

Net CP55,940-stimulated [^{35}S]GTP γ S binding in brain sections from cJun- OFF and cJun-ON mice following repeated vehicle or THC treatment.

Brain Region	Net CP55,940-stimulated [^{35}S]GTP γ S binding (nCi/g) $\pm$ SEM			
	cJun-OFF Vehicle	cJun-OFF THC	cJun-ON Vehicle	cJun-ON THC
Prefrontal Cortex	340 $\pm$ 16	185 $\pm$ 13***	355 $\pm$ 13	193 $\pm$ 12###
Nucleus Accumbens	227 $\pm$ 23	99 $\pm$ 19**	275 $\pm$ 36	155 $\pm$ 16##
Caudate-Putamen	114 $\pm$ 16	70 $\pm$ 17	153 $\pm$ 16	68 $\pm$ 16##
Globus Pallidus	535 $\pm$ 30	244 $\pm$ 36***	486 $\pm$ 44	279 $\pm$ 24###
Hippocampus	107 $\pm$ 9	19 $\pm$ 13***	118 $\pm$ 36	44 $\pm$ 23###
Amygdala	218 $\pm$ 16	62 $\pm$ 9***	282 $\pm$ 15**	100 $\pm$ 15###
VTA	274 $\pm$ 17	66 $\pm$ 7***	247 $\pm$ 18	79 $\pm$ 10###
Substantia Nigra	467 $\pm$ 29	260 $\pm$ 28***	479 $\pm$ 29	277 $\pm$ 29###
Cerebellum	250 $\pm$ 19	128 $\pm$ 11***	227 $\pm$ 24	126 $\pm$ 16###

Brain sections were assayed as described in Methods and autoradiograms were analyzed using densitometry. Results are expressed as net CP55,940-stimulated [^{35}S]GTP γ S binding (nCi/g) $\pm$ SEM, one-way ANOVA, Bonferroni post-hoc tests.

**
p < 0.01,

p < 0.001 vs. cJun-OFF vehicle.

p < 0.01,

p < 0.001 vs. cJun-ON vehicle. (n = 8–10 per group)

Table 3

$B_{\max}$ and K_D values from [^3H]SR141716A saturation binding analysis

	<u>cJun Off</u>		<u>cJun On</u>	
	$B_{\max}$ (pmol/mg)	K_D (nM)	$B_{\max}$ (pmol/mg)	K_D (nM)
CPu, Vehicle	2.19 ± 0.26	1.05 ± 0.35	2.56 ± 0.48	0.78 ± 0.19
CPu, THC	1.68 ± 0.38	1.48 ± 0.42	1.55 ± 0.42	0.62 ± 0.18
Amyg, Vehicle	2.83 ± 0.32	0.95 ± 0.10	2.16 ± 0.21	0.86 ± 0.27
Amyg, THC	2.24 ± 0.38	1.82 ± 0.42	2.57 ± 0.45	1.52 ± 0.47
Hipp, Vehicle	2.70 ± 0.38	0.56 ± 0.15	2.38 ± 0.24	0.45 ± 0.09
Hipp, THC	$1.34 \pm 0.16^*$	0.83 ± 0.17	$1.67 \pm 0.33^*$	0.73 ± 0.08

Data are mean values $\pm$ SEM from 5–6 mice per group. CPu, caudate-putamen; Amyg, amygdala; Hip, hippocampus.

* Main effect of THC treatment by two-way ANOVA.