Daily Δ⁹-Tetrahydrocannabinol and Withdrawal Increase Dopamine D₁-D₂ Receptor Heteromer to Mediate Anhedonia- and Anxiogenic-like Behavior Through a Dynorphin and Kappa Opioid Receptor Mechanism

Ahmed Hasbi, Bertha K. Madras, and Susan R. George

ABSTRACT

BACKGROUND: Frequent cannabis use is associated with a higher risk of developing cannabis use disorder and other adverse consequences. However, rodent models studying the underlying mechanisms of the reinforcing and withdrawal effects of the primary constituent of cannabis, Δ⁹-tetrahydrocannabinol (THC), have been limited.

METHODS: This study investigated the effects of daily THC (1 mg/kg, intraperitoneal, 9 days) and spontaneous withdrawal (7 days) on hedonic and aversion-like behaviors in male rats. In parallel, underlying neuroadaptive changes in dopaminergic, opioidergic, and cannabinoid signaling in the nucleus accumbens were evaluated, along with a candidate peptide designed to reverse altered signaling.

RESULTS: Chronic THC administration induced anhedonic- and anxiogenic-like behaviors not attributable to altered locomotor activity. These effects persisted after drug cessation. In the nucleus accumbens, THC treatment and withdrawal catalyzed increased cannabinoid CB₁ receptor activity without modifying receptor expression. Dopamine D₁-D₂ receptor heteromer expression rose steeply with THC, accompanied by increased calcium-linked signaling, activation of BDNF/TrkB (brain-derived neurotrophic factor/tropomyosin receptor kinase B) pathway, dynorphin expression, and kappa opioid receptor signaling. Disruption of the D₁-D₂ heteromer by an interfering peptide during withdrawal reversed the anxiogenic-like and anhedonic-like behaviors as well as the neurochemical changes.

CONCLUSIONS: Chronic THC increases nucleus accumbens dopamine D₁-D₂ receptor heteromer expression and function, which results in increased dynorphin expression and kappa opioid receptor activation. These changes plausibly reduce dopamine release to trigger anxiogenic- and anhedonic-like behaviors after daily THC administration that persist for at least 7 days after drug cessation. These findings conceivably provide a therapeutic strategy to alleviate negative symptoms associated with cannabis use and withdrawal.

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Biological focus was the dopamine D1-D2 receptor heteromer, which THC-induced functional and molecular changes. The initial exposure and withdrawal and presents a strategy to reverse impairments associated with cannabis use. We focused on reward sensitivity, negative emotionality, and other behavioral exposures resulted in D1-D2 heteromer upregulation, accompanied by reduced during spontaneous or antagonist-induced withdrawal in male rats treated with high THC doses for a few days (51,64).

This study investigated the consequences of chronic THC exposure and withdrawal and presents a strategy to reverse THC-induced functional and molecular changes. The initial focus was the dopamine D1-D2 receptor heteromer, which contributes to the aversive-, anhedonic-, depressive-, anxiogenic-like, and diminished motivational behaviors in rodents (65–68). In adult nonhuman primates, repeated THC exposure resulted in D1-D2 heteromer upregulation, accompanied by modulation of specific proteins involved in the D1-D2 heteromer signaling pathway (69), which include increased calcium signaling markers and decreased cAMP (cyclic adenosine monophosphate)-linked signaling proteins. As we postulated (69), these changes may contribute to reduced reward sensitivity, negative emotionality, and other behavioral impairments associated with cannabis use. We focused on defining changes occurring in the D1-D2 heteromer expression and function after chronic THC administration and following a withdrawal phase. We also determined whether D1-D2 heteromer disruption would reverse any of the behavioral or biochemical changes associated with chronic THC administration and withdrawal.

METHODS AND MATERIALS

Animals

Adult male Sprague Dawley rats (300–325 g) were used in compliance with the guidelines from the Canadian Council on Animal Care.

Experimental Design

The experiments were designed and conducted as shown in Figure 1.

Sucrose Preference Test. Rats were presented with a 2-bottle choice: sucrose (1.5% wt/vol) or water for 2 hours (Figure 2).

Open Field Test. The experiments were performed as previously described (70). Multiple parameters were recorded and analyzed including total distance traveled, time spent in and entries to the center zone compartment of the chamber, vertical activity, and ambulatory activity times (Figure 3).

Light/Dark Box Test. The test was performed as previously described (70), with only minor modifications. The rats were allowed to freely explore the dark and lighted compartments for 5 minutes. Analyses included the time spent in the light versus the dark side of the box and the number of re-entries/retreats during the 5-minute test.

Western Blotting. Multiple proteins from the cytosol and membrane were analyzed on the same gels using Odyssey imaging system (LI-COR Biosciences). Proteins were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose or polyvinylidene fluoride membranes. Membranes were subject to immunoblotting and exposed to secondary antibody labeled with infrared fluorescence dyes (LI-COR Biosciences). Odyssey scanner was used for imaging.

Proximity Ligation Assay. In situ proximity ligation assay was performed to assess D1-D2 receptor complexes as described previously (86,89).

Grooming Test. Rats were injected with SKF 83959 after pretreatments with control or active peptides or with KOR antagonist and placed in their home cages. After a 5-minute delay, grooming behavior was registered every 15 seconds over a total period of 30 minutes.

Statistical Analysis

Statistical analyses were performed using the Prism software (version 9.3; GraphPad Software). The type of statistical test conducted is indicated with each result. See Detailed Materials and Methods in the Supplement.

RESULTS

Chronic THC and Withdrawal Modulate Dopamine D1-D2 Heteromer Expression

In accordance with a study in nonhuman primates (69), daily THC (1 mg/kg, intraperitoneal [i.p.], 9 days) increased the number of D1-D2 proximity ligation assay signals/mm² (one-way analysis of variance [ANOVA], F3,20 = 36.45, p < .0001; vehicle vs. THC, p = .008) and the number of heteromer-expressing cells (i.e., DAPI-labeled nuclei) in the ventral striatum (i.e., NAc) (Figure 2) (one-way ANOVA, F3,20 = 85.5, p < .0001; vehicle vs. THC, p < .0001) and the dorsal striatum (Figure 3) (proximity ligation assay signals/mm²: one-way ANOVA, F3,20 = 108.9, p < .0001; heteromer-expressing cells: one-way ANOVA, F3,20 = 16.76, p < .0001). The increase persisted after the 7-day withdrawal phase (withdrawal+TAT-scrambled peptide [WD+TAT-Sc]) in both striatal subregions (Figures 2 and 3), but it was abolished in animals treated with TAT-D1 during the same withdrawal phase (Figures 2 and 3). The treatment with TAT-D1 also decreased the D1-D2 heteromer density in comparison to vehicle-treated animals, signifying that intranasal administration of TAT-D1 enabled access of the peptide to the striatum, as also confirmed by fluorescent-dansyl-TAT-D1 peptide localization (Figure S1).
Behavioral Assessment of Chronic THC Administration and Withdrawal

Owing to the involvement of the D₁-D₂ heteromer in aversion, anxiety, addiction, and depression (65–67), we evaluated the effects of daily THC administration and withdrawal on reward- or aversion- and anxiety-linked behaviors (according to the experimental timeline shown in Figure 1) and whether the D₁-D₂ heteromer would be associated with a specific behavior.

Sucrose Preference Test. During baseline assessment (T1), rats presented with a 2-bottle choice of water and 1.5% sucrose showed a pronounced preference (88.6% ± 1.23%) for the sucrose solution (Figure 4A), which declined significantly after the 9-day THC treatment (Figure 4B) (one-way ANOVA, F_{2,69} = 67.37, p < .0001; basal vs. THC, q = 16.17), but it was unchanged in the vehicle-treated group (basal vs. vehicle, q = 2.7). In the subgroup that received the D₁-D₂ heteromer disrupting peptide during the withdrawal phase (WD + TAT-D₁), repeated-measures ANOVA (F_{2,17} = 77.2, p < .0001) confirmed the THC-induced decrease in sucrose preference (basal vs. THC, p < .0001), an effect countered by TAT-D₁ during withdrawal (Figure 4C) (THC vs. WD + TAT-D₁, p < .0001). In contrast, in the cohort that received the control-ineffective peptide (WD + TAT-Sc) (Figure 4D) (repeated-measures ANOVA, F_{2,17} = 6.99, p = .001), the THC-induced decrease in sucrose consumption (basal vs. THC, p = .04) was not restored after the withdrawal phase (THC vs. WD + TAT-Sc, p = .83; basal vs. WD + TAT-Sc, p = .015). These results were confirmed by calculating the differences in sucrose consumption after the 9-day THC treatment (T2 – T1) and the 7-day withdrawal period (T3 – T2) for each of the 2 groups receiving the peptides (WD + TAT-D₁ and WD + TAT-Sc). Two-way ANOVA (Figure 4E) revealed significant effect of treatment (F_{1,28} = 43.3, p < .0001) and significant interaction between treatment and the peptide used (F_{1,28} = 17.9, p = .0004). Tukey’s multiple comparisons test showed that chronic THC treatment had a similar decreasing effect in both groups (THC/WD + TAT-D₁ vs. THC/WD + TAT-Sc, p = .19) and that TAT-D₁ reversed the THC effect (THC/TAT-D₁ vs. WD + TAT-D₁, p < .0001) in contrast to TAT-Sc (THC/TAT-Sc vs. WD + TAT-Sc, p = .17).

These observations suggested that daily THC induced anhedonia-like behavior that persisted after the 7-day drug withdrawal period. D₁-D₂ heteromer disruption decreased the anhedonia-like symptom observed after spontaneous withdrawal, conceivably linking THC-induced elevation in D₁-D₂ expression to THC-induced aversive-like effect.

Open Field Test. This test was used to assess the effects of chronic THC and withdrawal on locomotor and exploratory behavior in accordance with data from other groups (70) (Figure 5; Figure S4). None of the parameters relative to exploratory or locomotor activities were significantly affected by the treatments. Basal locomotor activity (T1: 2609 ± 114 cm/min) (Figure S4A) significantly decreased (Figure 5A; Figure S4A1) (one-way ANOVA, F_{2,69} = 8.53, p = .0005) after the 9-day treatment with vehicle (basal vs. vehicle, p = .022) and THC (basal vs. THC, p = .0009; vehicle vs. THC, p = .68), possibly reflecting adaptation to the environment (from test-1:T1 to test-2:T2). Treatment with either TAT-D₁ or control TAT-Sc peptides during the 7-day withdrawal phase had no significant effect on locomotion (Figure 5A; Figure S4A2).

Similarly, the center zone parameters were not affected by any treatment. There were no significant differences in the latency to enter (one-way ANOVA, F_{4,24} = 0.25, p = .92) (Figure S5b1), number of entries (one-way ANOVA, F_{4,24} = 2.16, p = .08) (Figure S5b2), duration (one-way ANOVA, F_{4,24} = 1.42, p = 2.4) (Figure S5b3), and distance traveled in the center zone (one-way ANOVA, F_{4,24} = 1.85, p = .13) (Figure S5b4).

Light/Dark Chamber Test. In a test designed to detect anxiogenic-/anxiolytic-like behavior, rats spent approximately 75% of the 5-minute test period (T1) in the dark side of the chamber (187 ± 5.4 seconds) (Figure 6A, B) (basal) in accordance with the known preference of rodents for darkened environments. The rats treated daily with THC (T2) spent more time in the dark side of the chamber compared with the time they spent before drug treatment (Figure 6b) (paired t test with Welch’s correction, p < .0001, t₁₁₆ = 5.55). In contrast, vehicle-treated control rats showed no changes in light/dark preference (Figure 6A) (paired t test with Welch’s correction, p = .10, t₁₁₆ = 1.73). Indeed, in direct comparison to
vehicle treatment, the 9-day THC treatment increased time spent in the dark box (Figure 6C) (unpaired t test, $p = .0001$, $t_{27.46} = 5.34$).

In the subgroup treated with the control TAT-Sc peptide (Figure 6D), repeated-measures ANOVA showed no significant effect ($F_{2,17} = 18.3, p = .09$) in reversing the THC-induced preference for the dark box (basal vs. THC, $p = .04$), which persisted despite the 7-day drug withdrawal (THC vs. TAT-Sc, $p = .3$). In contrast, TAT-D1 peptide (Figure 6E) significantly reversed (repeated-measures ANOVA, $F_{2,17} = 18.3, p = .0003$) the THC-induced increase in the time spent in the dark compartment (basal vs. THC, $p = .001$; THC vs. TAT-D1, $p = .005$).

These observations were strengthened by another assessment of anxiogenic-like behavior, namely the number of times the rats emerged from the dark side (70). Repeated-measures ANOVA applied to the WD+TAT-Sc group showed that treatment had an overall significant effect ($F_{2,17} = 9.6, p = .008$), with the 9-day THC treatment decreasing the number of emergences (basal vs. THC, $p = .038$) (Figure 6F), which persisted after the 7-day withdrawal period in animals treated with the control peptide (THC vs. TAT-Sc, $p = .28$) (Figure 6F). In contrast, repeated-measures ANOVA ($F_{2,17} = 9.9, p = .01$) in the WD+TAT-D1 group (Figure 6G) showed that while THC had similar effect in lowering the number of emergences from the dark side (basal vs. THC, $p = .045$) (Figure 6G), treatment
with TAT-D1 reversed the reduced emergence from the dark compartment (THC vs. WD+TAT-D1, \( p = .03 \)) (Figure 6G). Because the number of increased passages of dark-to-light side is associated with improvement of anxiogenic-like behavior (70), the results are suggestive of an increased anxiety-like/fearfulness-like behavior following a 9-day THC treatment regimen persisting after spontaneous withdrawal, unless with concomitant disruption of the D1-D2 heteromer, which relieved the fear- or anxiety-like behavior.

Molecular Adaptations in Chronic THC–Treated Animals and Withdrawal From THC

THC induces significant dopamine release in the terminal projection regions of the mesocorticolimbic neurons originating in the ventral tegmental area, including in the NAc (32,33,51,58). Because the NAc represents the central hub of this circuitry underlying reward mechanisms, analyses were focused on changes occurring in this region. Multiple proteins involved in the signaling pathways of D1-D2 heteromer in anhedonia, anxiety, aversion, and drug addiction were selectively targeted using Western blotting analysis.

CB1R Activation. As mentioned, CB1R mediates most THC effects. The expression of CB1R and its phosphorylation status were analyzed (Figure 7A). Compared with vehicle-treated animals, total CB1R expression was not affected (one-way ANOVA, \( F_{1,24} = 0.6, p = .65 \)) by repeated THC administration or after 7 days of drug withdrawal. However, the ratios of phosphorylated CB1R to total CB1R
Dopamine D₁ and D₂ Receptors. In addition to the increase in D₁-D₂ heteromer (Figures 4 and 5), THC increased dopamine D₁ receptor (D₁R) expression in comparison to the vehicle group (one-way ANOVA, F₁,24 = 12.03, p < .0001) (Figure 7B) (vehicle vs. THC, p = .0009), which remained high during withdrawal in animals treated with the control peptide (vehicle vs. WD + TAT-Sc, p < .0001), but returned to vehicle-treated levels in animals treated with the TAT-D1 peptide (vehicle vs. WD + TAT-D1, p = .04). D₂R expression decreased after repeated THC treatment compared with vehicle treatment (Figure 7B, right panel) (one-way ANOVA, F₁,24 = 3.08, p = .03; vehicle vs. THC, p = .03), but returned to levels comparable to that in vehicle-treated animals after drug withdrawal (vehicle vs. WD + TAT-Sc, p = .68). TAT-D1 treatment during withdrawal maintained the reduction in D₂R expression (vehicle vs. WD + TAT-D1, p = .04).

Calcium/Calmodulin-Dependent Protein Kinase IIα. Calcium-mediated activation of CaMKIIα (calcium/calmodulin-dependent protein kinase IIα) occurs through phosphorylation at Thr286 (pCaMKIIα) following chronic THC treatment in nonhuman primates (69). Treatment had a significant effect on pCaMKIIα (one-way ANOVA, F₁,24 = 92.8, p < .0001) (Figure 8A, left panel) (vehicle vs. THC, p < .0001). This effect was reversed after the 7-day withdrawal period whether rats received TAT-D1 (vehicle vs. WD + TAT-D1, p = .98) or the control peptide (vehicle vs. WD + TAT-Sc, p = .18).

Tropomyosin Receptor Kinase B. BDNF/TrkB signal activation is known to be involved in D₁-D₂ heteromer function (71), and it is regulated following chronic THC administration in nonhuman primates (69). The 9-day treatment with THC and the 7-day withdrawal phase did not affect the total expression level of TrkB (Figure 8B, left panel) (F₁,24 = 2.49, p = .07). However, the phosphorylation of TrkB (pTrkB) relative to total TrkB [ratio, pTrkB/TrkB] revealed a significant modulation of the ratio [pTrkB/TrkB] by the different treatments (one-way ANOVA, F₁,24 = 13.03, p < .0001) (Figure 8B, right panel), with daily THC treatment increasing this ratio (vehicle vs. THC, p = .02), which subsequently decreased after withdrawal (THC vs. WD + TAT-Sc, p < .0001; THC vs. WD + TAT-D1, p = .015).

cAMP-PKA-DARPP-32 Pathway. The cAMP-PKA-DARPP-32 signaling pathway, usually activated after acute drug intake (74), was silent because no effect of treatment was
observed in the phosphorylation of ERK (Figure 8C, left panel) (one-way ANOVA, $F_{4,24} = 1, p = .42$) nor in the AMPA receptor GluA1-subunit at Ser845 (pGluA-S845), a target of the cAMP-PKA signaling pathway (Figure 8C, right panel) (one-way ANOVA, $F_{4,24} = 0.3, p = .87$). Although there was a significant effect of treatment on Thr-34-DARPP-32 phosphorylation (Figure 9A, left panel) (one-way ANOVA, $F_{4,24} = 15.03, p = .0001$), the following multiple comparisons test showed that daily THC treatment and withdrawal had no effect and that in fact TAT-D1 treatment increased pT34-DARPP-32 compared with the vehicle ($p = .001$), THC ($p = .004$), and control (WD+TAT-Sc, $p < .0001$) groups. Phosphorylation of DARPP-32 at Thr-75 (pT75-DARPP-32) was unchanged in all groups (Figure 9A, right panel) (one-way ANOVA, $F_{4,24} = 2.17, p = .10$).

Akt/GSK-3 Pathway. Chronic (21 days) THC dephosphorylated (i.e., activated) GSK-3 in nonhuman primates (69). However, in rats, the shorter period of treatment with THC (9 days) had no significant effect on GSK-3 phosphorylation (one-way ANOVA, $F_{4,24} = 1.02, p = .42$) (Figure 9B) or on one of its modulators, Akt at Thr308 ($F_{4,24} = 0.66, p = .63$) and Ser473 ($F_{4,24} = 1.48, p = .24$).

Linking THC-Induced Effects to D1-D2 Heteromer Function and Opioid Dynorphin/KOR Action

Our results thus far suggested that an increase in D1-D2 heteromer expression and signaling may be directly involved in the aversive effects of chronic THC and drug withdrawal. We
DA D1-D2 Heteromer Mediates THC Effects Through Dyn/KOR

Figure 6. Light/dark box anxiety test. Effects of chronic treatment (daily intraperitoneal injection for 9 days) with vehicle (A) or THC (1 mg/kg) (B) on the time spent in the dark compartment (t tests, **p ≤ .01, ns p > .05). (C) Comparison of the effects of vehicle vs. THC treatments on the time spent in the dark side (t test, ***p ≤ .001). (D, E) Analysis of the effects of the different treatments on the time spent in the dark compartment in the groups that went through withdrawal and received the control TAT-Sc peptide (D) or the TAT-D1 peptide (E). Repeated-measures analyses of variance, *p ≤ .05, **p ≤ .01, ns p > .05. (F, G) Analysis of the effects of the different treatments on the number of passages from the dark to the lighted side (emergences) in the groups that went through withdrawal and received the control TAT-Sc peptide (F) or the TAT-D1 peptide (G). Repeated-measures analyses of variance, *p ≤ .05, **p ≤ .01, ns p > .05. n = 6 rats per condition. Error bars indicate SEM. Basal refers to activity of all subjects prior to initiation of experimental procedures. ns, not significant; Sc, scrambled; THC, Δ9-tetrahydrocannabinol; WD, withdrawal.

postulated that these effects may be mediated in part by the dynorphin/KOR pathway. Overall, dynorphin expression was altered by treatment (one-way ANOVA, F4,24 = 5.9, p = .002). Repeated THC administration significantly elevated dynorphin levels (Figure 10A) (vehicle vs. THC, p = .06), which persisted after the 7-day withdrawal period (THC vs. WD+TAT-D1, p = .9; THC vs. WD+TAT-Sc, p = .7). THC treatment had no effect on total KOR expression (Figure 10B, left panel) (one-way ANOVA, F4,24 = 0.8, p = .53). However, analyzing the ratios of phosphorylated KOR (pKOR) and total KOR (pKOR:KOR) (Figure 10B, right panel) (one-way ANOVA, F4,24 = 16.2, p < .0001) showed that the 9-day treatment with THC increased the [pKOR:KOR] ratio significantly (vehicle vs. THC, p < .0001), which was restored to vehicle-treated values after withdrawal in both the TAT-D1 (vehicle vs. WD+TAT-D1, p = .83) and the TAT-Sc (vehicle vs. WD+TAT-Sc, p = .99) groups.

To assess a probable direct link between D1-D2 heteromer activation and the dynorphin/KOR pathway, we proceeded as follows. First, direct activation of D1-D2 by SKF 83959 administration (0.5 mg/kg subcutaneous, 4 hours) modified the dynorphin content in the NAc (Figure 11A). Three dynorphin forms were documented on Western blotting, prodynorphin (ProDyn) species of molecular weights w45 and w25 kDa and another of approximately 17 kDa, known as big dynorphin (Big Dyn). Analysis showed that the ProDyn species decreased significantly (ProDyn 45 kDa, p = .031, t test) or tended to decrease (ProDyn 25 kDa, p = .18, t test), whereas big dynorphin, more commonly known to reflect released dynorphin, was significantly increased after SKF
83959 treatment (p = .006, t test). Owing to its aversive and proanxiogenic-like effects (66), SKF 83959 led to increased self-grooming (Figure 11B) (one-way ANOVA, $F_{3,20} = 27.7, p < .0001$), an effect due to D1-D2 heteromer activation because the TAT-D1 peptide, but not the control peptide, was able to inhibit the SKF 83959–induced grooming. Interestingly, pretreatment with the KOR antagonist, norbinaltorphimine (10 mg/kg, i.p., 24 hours), inhibited the SKF 83959–induced and D1-D2 heteromer–mediated grooming (Figure 11C) (one-way ANOVA, $F_{2,18} = 38.2, p < .0001$). Collectively, these data suggested that D1-D2 heteromer activation mediated the SKF 83959–induced aversive- and anxiogenic-like effects through a mechanism involving increased dynorphin and stimulation of the KOR pathway.

**DISCUSSION**

We report a novel mechanism linking the aversive-like behaviors and changes in neuronal signaling following repeated THC and spontaneous withdrawal to interactions between cannabinoid, dopamine, and opioid systems, involving, in part, an elevation in dopamine D1-D2 heteromer expression and function.

Numerous neuroadaptive changes affecting the 3 neurotransmitter systems, dopamine, cannabinoid, and opioid, occurred at the molecular level. In contrast to the unmodified CB1R expression, its activity, reflected by its phosphorylation state, tended to increase after repeated THC treatment and was heightened after drug withdrawal, probably indicating an activation of the endocannabinoid system. Similar to many G protein–coupled receptors, phosphorylated CB1R may undergo desensitization, internalization, and/or downregulation following chronic exposure to cannabinoids (75–78). In humans, CB1R downregulation was shown to be correlated with duration of cannabis smoking and was reversible after approximately 4 weeks of cannabis abstinence (79), but it was regionally specific, with significant downregulation occurring selectively in the cortical brain regions, but not observed in the caudate, putamen, or ventral striatum (NAc) (79). This latter result is in line with the present data showing no change in total CB1R expression in the NAc of male rats.
after 9 days of THC treatment. Interestingly, the increased CB1R activity that persisted after the withdrawal phase was reduced to control levels by disrupting the D1-D2 heteromer, thus linking the sustained activation of the endocannabinoid system to dopaminergic system through the D1-D2 heteromer activity.

Indeed, repeated THC-CB1R activation increased the density of D1-D2 heteromer and the number of heteromer-expressing cells in the striatum, an effect sustained after drug withdrawal, except in animals treated with TAT-D1 peptide, indicating successful specific D1-D2 heteromer disruption by intranasal TAT-D1 peptide administration. In parallel, daily THC increased D1R and decreased D2R expression in the NAc. Reduced D2R availability was reported in human misusers of stimulants, alcohol, and cocaine and in long-time marijuana users (8,9,80,81). Thus, recurrent exposure to drugs that overstimulate dopamine signaling may lead to decreased D2R in line with the decrease in D2R messenger

Figure 8. THC-induced neuroadaptive changes in signaling proteins: pCaMKII and pTrkB. Western blotting analysis of homogenates from rats that received chronic treatment (daily intraperitoneal injection for 9 days) with vehicle or THC (1 mg/kg). Groups of rats went through drug withdrawal for 7 days, during which they received daily intranasal TAT-D1 peptide (WD+ TAT-D1) or a control TAT-Sc peptide (WD+ TAT-Sc). (A) Analysis of the phosphorylation of CaMKII at Thr286. (B) Analysis of TrkB total expression relative to GAPDH (left panel) and ratio of phosphorylated TrkB to total TrkB (pTrkB:TrkB) (right panel). (C–D) Analysis of the phosphorylation of ERK (C) and AMPA receptor Ser845-GluA1 (D) in the different groups. Data are mean ± SEM. n = 6 rats per group. One-way analyses of variance with multiple comparisons: *p < .05, **p < .001; p > .05 (not significant). ERK, extracellular signal-regulated kinase; pCaMKIIα, phosphorylated CAMKIIα; pERK, phosphorylated ERK; Sc, scrambled; THC, Δ2-tetrahydrocannabinol; pTrkB, phosphorylated TrkB; TrkB, tropomyosin receptor kinase B; Veh, vehicle; WD, withdrawal.
RNA (69) or D2R protein (this study) after chronic THC administration.

Other important neuroadaptations were observed in the signaling pathways such as increased BDNF/TrkB signaling, activation of calcium/CaMKII pathway but not the cAMP/PKA/DARPP-32-related pathway, in line with previous studies in nonhuman primates (69). Drugs of abuse are known to acutely activate D1R-Gs/olf pathway leading to cAMP accumulation, PKA activation, Thr-3-DARPP-32 phosphorylation, and protein-phosphatase-I inhibition (74). This suggests that increased heteromer density may function initially to decrease this superactivated reward pathway as was shown in a cocaine administration model (66). However, prolonged/repeated D1-D2 activation induces aversion and anhedonia because of its reward inhibitory effects (66). To counter these negative effects, a reduction in D2R expression may then occur to balance excitatory versus inhibitory dopamine signaling. Taken together, these observations implicate an important physiological regulatory role for the D1-D2 heteromer in the NAc by modulating the balance between D1- versus D2 receptor–mediated signaling pathways to maintain hedonic equilibrium.
Another interesting result is the activation of the calcium-dependent pathway manifested by CaMKII\(\alpha\) activation and BDNF/TrkB signaling, both of which are part of the well-documented D1-D2-linked calcium signal (71), an effect similar to that elicited by chronic THC in adult rhesus monkeys (69), which indicates that repeated THC may activate, in part, calcium-CaMKII\(\alpha\) and BDNF/TrkB signaling through increased D1-D2 heteromer expression/activation. Elevated BDNF-TrkB activity in the NAc contributes to depressive-/anhedonic-like behaviors in rats (82) and was observed after escalating marijuana use among adolescents and also in adults with CUD (83,84), with dynorphin being proposed as a downstream BDNF effector in the striatum (83–86). Intriguingly, increased dynorphin expression and enhanced phosphorylation of its receptor KOR were observed following repeated THC treatment and withdrawal, adding thus another layer of interaction between the 3 important systems. The findings support a link between repeated cannabinoid system activation, upregulation of expression and activity of dopamine D1-D2 heteromer, and increased dynorphin/KOR signaling, a system associated with dysphoria and aversion. The linkage between dopamine receptor heteromer activity and upregulation of dynorphin/KOR signaling was reinforced by the demonstration that direct activation of the D1-D2 heteromer by an agonist resulted in increased dynorphin expression in the NAc and led to increased self-grooming, a manifestation of self-soothing behavior attempting to alleviate increased anxiety and dysphoria in rodents. The increased grooming was blocked by the TAT-D1 peptide and, more importantly, by administration of the KOR antagonist nor-binaltorphimine, clearly involving the dynorphin/KOR system in the D1-D2 heteromer-mediated aversive effect.

Taken together, we propose a novel mechanism underlying the aversion- and anxiogenic-like behaviors after repeated THC exposure and withdrawal that associates the dopamine D1-D2 heteromer neurons in the NAc to cannabinoid, dopamine, and opioid signaling cascades (Figure 12). According to the literature (87–89), a single exposure to THC activates CB1R

![Figure 10. THC-induced changes in opioid peptides and the KOR. Western blotting analysis of homogenates from rats that received chronic treatment (daily intraperitoneal injection for 9 days) with vehicle or THC (1 mg/kg). Groups of rats went through drug withdrawal for 7 days, during which they received daily intranasal TAT-D1 peptide (WD+TAT-D1) or control TAT-Sc peptide (WD+TAT-Sc). (A) Analysis of the expression of Dyn in the different groups compared with GAPDH. A form of 17–18 kDa, representing big dynorphin, usually called dynorphin, is shown. (B) Analysis of the total expression and phosphorylation of KOR in the different groups. Data are mean ± SEM, n = 6 rats per group. One-way analyses of variance with multiple comparisons: \(p \leq .05, * p \leq .01, ** p \leq .0001; p > .05 \) (not significant). Dyn, dynorphin; KOR, kappa opioid receptor; pKOR, phosphorylated KOR; Sc, scrambled; THC, Δ9-tetrahydrocannabinol; Veh, vehicle; WD, withdrawal.}
to inhibit the GABAergic input (Figure 12, step 1), leading to increased dopamine release (Figure 12, step 2). Repeated THC exposure, however, elevates D1-D2 heteromer density (Figure 12, step 3), which is sustained after spontaneous withdrawal for 7 days. Dopamine activity at the D1-D2 heteromer would activate the well-known (71) Gq-mediated increased calcium mobilization and activation of calcium-linked signaling cascades (Figure 12, step 4) including increased CaMKII activity and BDNF expression (Figure 12, step 5). In analogy with the biochemical cascade triggered by cocaine action (90,91), BDNF/TrkB activation would lead to increased CREB (cAMP response element binding protein) activation and ProDyn synthesis and processing (Figure 12, step 6). Alternatively, BDNF can activate TrkB on all medium spiny neuron types (66,92,93), resulting in increased dynorphin release from D1 medium spiny neurons and D1/D2 medium spiny neurons (Figure 12, step 6). Dynorphin would activate its receptor, KOR (94), present on presynaptic dopamine neurons (Figure 12, step 7). This would lead to increased dopamine release in the NAcc after chronic drug treatment (Figure 12, step 8), which would contribute to the anhedonia- and anxiogenic-like behavior observed in this study (Figure 12, step 9) and to

![Figure 11.](image)

**Figure 11.** Link between THC-induced signaling cascades, dynorphin/kappa opioid receptor activation, and dopamine D1-D2 heteromer function. (A) Analysis of the levels of prodynorphin-derived peptides (45, 25, and 17 kDa) in the nucleus accumbens of rats treated with SKF 83959 (0.5 mg/kg, subcutaneous) for 4 hours. Western blotting reveals 2 isoforms of prodynorphin, 48 and 25 kDa, and one form of 17–18 kDa, representing big dynorphin, usually called dynorphin. Analyses of each band intensity relative to GAPDH are summarized. Data are means ± SEM. n = 3 rats per group. *p < .05, **p < .01, ***p < .001, ns p > .05. (B) Selfgrooming behavior was analyzed in rats treated with SKF 83959 (0.5 mg/kg, subcutaneous, 4 hours prior) after intranasal pretreatment with the inactive (TAT-Sc) and active (TAT-D1) peptides (15 µg, 30 minutes prior). (C) SKF 83959–induced grooming following treatment with the kappa opioid receptor antagonist nor-BNI (10 mg/kg, intraperitoneal, 24 hours prior). Data are mean ± SEM. n = 6 rats per group. One-way analyses of variance with multiple comparisons: *p < .05; **p < .01; ***p < .001; ns, p > .05. BNI, binaltorphimine; Dyn, dynorphin; MW, molecular weight; ns, not significant; ProDyn, prodynorphin; Sc, scrambled; Veh, vehicle.
the general aversion-like effect after chronic drug treatment and repetitive activation of the D1-D2 heteromer (66). The presented schematic model is simplified and condensed to facilitate the presentation and interpretation of the D1-D2 heteromer–related signaling cascades in the NAc. Although we narrow the focus of this mechanism based on our empirical data, other potential contributors include other neurotransmitter/receptor signaling systems, other types of cells, such as interneurons and glial cells, and different brain regions and circuit nodes involved in aversion-anhedonia.

As in any study, there are limitations and caveats. One major caveat is the use of only male adult rats. We anticipate that there would be many differences because of sex and other factors such as age in the cannabinoid, dopaminergic, and opioidergic systems. Especially, significant sex (95) and age (73) differences in D1-D2 expression in the NAc were documented, and ongoing studies are examining the effects of THC in these populations.

One interesting result in this study is that disrupting D1-D2 heteromer activity during withdrawal fostered remission from the observed anhedonia- and anxiogenic-like behaviors. Thus, the dopamine D1-D2 heteromer may represent the first discrete molecular mechanism identified that is activated after repeated THC and which, if interrupted, reverses the behavioral and biochemical manifestations of drug withdrawal. Clinically, the prevalence of cannabis withdrawal symptoms has been reported to occur in up to 47% to 95% of heavy users (96–99).

Because the withdrawal symptoms in human cannabis users are catalysts for ongoing drug seeking and relapse, this novel strategy should be further evaluated for providing symptom relief and a stabilizing effect to remain in treatment for CUD.

In summary, we identified a unique molecular target that can be activated to inhibit the rewarding effects of drugs of abuse (66) or could be inhibited to alleviate the negative symptomatology during drug withdrawal (this study), which may be a potent stimulus for continued drug seeking. We also identified the efficacy of a selective peptide targeting the D1-D2 heteromer, which when administered by the intranasal route, reversed the associated neurochemical and behavioral changes. These significant, potentially highly translatable findings provide a novel pharmacotherapeutic strategy not only to alleviate negative withdrawal symptoms associated with CUD and possibly other substance use disorders but also to conceivably prevent relapse, thereby targeting the enormous unmet needs of compulsive motivational addiction states for which currently there are no effective treatments.

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ARTICLE INFORMATION
From the Department of Pharmacology and Toxicology (AH, SRG) and Department of Medicine (SRG), Temerty Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada; Laboratory of Addiction Biology (BK), McLean Hospital, Belmont; and the Department of Psychiatry (BKM), Harvard Medical School, Boston, Massachusetts.

Address correspondence to Ahmed Hasbi, Ph.D., at a.hasbi@utoronto.ca, or Susan R. George, M.D., at s.geroge@utoronto.ca.

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