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PII: S2667-1743(22)00088-X
DOI: https://doi.org/10.1016/j.bpsgos.2022.07.003
Reference: BPSGOS 156

To appear in: Biological Psychiatry Global Open Science

Received Date: 7 February 2022
Revised Date: 28 June 2022
Accepted Date: 7 July 2022

Please cite this article as: Hasbi A., Madras B.K. & George S.R., Daily THC and withdrawal increase dopamine D1-D2 receptor heteromer to mediate anhedonia and anxiogenic-like behavior through a dynorphin and kappa opioid receptor mechanism, Biological Psychiatry Global Open Science (2022), doi: https://doi.org/10.1016/j.bpsgos.2022.07.003.

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Daily THC and withdrawal increase dopamine D1-D2 receptor heteromer to mediate anhedonia and anxiogenic-like behavior through a dynorphin and kappa opioid receptor mechanism

Ahmed Hasbi1,#, Bertha K. Madras3 and Susan R. George1,2,#

1Department of Pharmacology and Toxicology, Temerty Faculty of Medicine, University of Toronto, Toronto, ON, Canada
2Department of Medicine, Temerty Faculty of Medicine, University of Toronto, Toronto, ON, Canada
3McLean Hospital, Belmont MA, USA and Department of Psychiatry, Harvard Medical School, Boston MA

#corresponding authors:
s.george@utoronto.ca
a.hasbi@utoronto.ca

Short Title: DA D1-D2 heteromer mediates THC effects through Dyn/KOR

Word count:
ABSTRACT (250 words)
Total text: 4284

INTRODUCTION (499 words)
METHODS AND MATERIALS: 298 words
RESULTS (2152 words)
DISCUSSION (1334 words)
ABSTRACT (250 words)

**Background:** Frequent cannabis use is associated with a higher risk of developing cannabis use disorder (CUD) and other adverse consequences. In rodents, modeling the underlying mechanisms of the reinforcing and withdrawal effects of the primary constituent of cannabis, Δ⁹-tetrahydrocannabinol (THC), has been limited.

**Methods:** The effects of daily THC (1mg/kg, i.p., 9 days) and spontaneous withdrawal (7 days) in male rats on hedonic and aversion-like behaviors were investigated. In parallel, underlying neuroadaptive changes in dopaminergic, opioidergic and cannabinoid signaling in the nucleus accumbens (NAc) 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 NAc, THC treatment and withdrawal catalyzed increased 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 brain-derived neurotrophic factor pathway (BDNF/TrkB), dynorphin expression and kappa opioid receptor (KOR) 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.

**Conclusion:** Chronic THC increases NAc dopamine D1-D2 receptor heteromer expression and function, which results in increased dynorphin expression and KOR 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.
INTRODUCTION

Cannabis is the most used illicit substance worldwide (1). Due to potential medicinal properties of cannabinoids, notably Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), some countries made substantial changes to relax cannabis regulations despite multiple reports of harmful consequences of cannabis use, notably in adolescents (2-20). In addition, the addictive potential of cannabis in humans is well-established and may lead to cannabis and substance use disorders (CUD/SUD, 21-23). Animal models for the reinforcing and withdrawal effects of THC are limited due to multiple issues (24-27). Still, they offer the advantage of controlled dose/frequency research in the absence of confounds encountered in human research (28-31).

The cannabinoid CB1 receptor (CB1R) mediates both the rewarding and aversive effects of THC (32-34), as well as tolerance (35), and physical dependence (36-37). Some neuroadaptive changes following chronic THC exposure include modifications of CB1R availability, desensitization, degradation, and a decrease in G-protein expression or activity (38). Cannabinoids engage not only the endogenous cannabinoid system, but opioid, dopamine, glutamate, GABA and other signaling systems (43-46). In rodents, CB1R function has a reciprocal relationship with opioids (47-53). For example, in CB1R gene-deleted mice, morphine-induced conditioned place preference (CPP), a measure of drug rewarding effects, was blocked and the severity of morphine withdrawal was attenuated (54-56). Mu (MOR), but not kappa (KOR) or delta (DOR) opioid receptor, has been implicated in THC-induced CPP, whereas KOR contributed to THC-induced conditioned place aversion (CPA, 49-52). The cannabinoid system has also reciprocal interactions with the dopamine system. In humans, long-term cannabis users manifest reduced dopamine release in the striatum (8-9, 57), accompanied by deficits in dopamine-related functions, leading to poor working memory, negative emotionality, impaired executive function, apathy, and lack of motivation (8-9). In preclinical studies, acute administration of THC or WIN 55,212-2, produced dose-dependent activation of mesolimbic dopaminergic neurons, through CB1R (58), which led to enhanced dopamine release in the nucleus-accumbens (NAc)-shell (59), observations confirmed in some preclinical and human studies (60-63; 9; 51). In contrast, dopamine activity was reduced during spontaneous or antagonist-induced withdrawal in male rats treated with high THC doses for few days (51; 64).
The present study investigates the consequences of chronic THC exposure and withdrawal and presents a strategy to reverse THC-induced functional and molecular changes. The initial focus is 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), that include increased calcium signaling markers and decreased cyclic-AMP (cAMP)-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 3A.

Sucrose preference test: Rats were presented with a two-bottle choice: sucrose (1.5%, w/v) or water for two hours.

Open field test: The experiments were performed as previously described (72). 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.

Dark/Light chamber test: The test was performed as previously described (72), 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 blot: Multiple proteins from the cytosol and membrane were analyzed on the same gels using Odyssey technology (LI-COR Biosciences). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose or PVDF 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 (PLA): In situ PLA was performed to assess D1-D2 receptor complexes as described previously (66; 69).

Grooming test: Rats were injected with SKF 83959 after pre-treatments 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 with Prism software (Prism 9.3). The type of statistical test conducted is indicated with each result.

Detailed Materials and Methods section is attached as Supplemental information.
RESULTS

Chronic THC and withdrawal modulate dopamine D1-D2 heteromer expression

In accordance with a study in non-human primates (69), daily THC (1 mg/kg, i.p., 9 days) increased the number of D1-D2 PLA signals/mm² (One-way ANOVA, \(F_{(3,20)}=36.45; p<0.0001\); Veh vs THC, \(p=0.008\)), and the number of heteromer-expressing cells (i.e. DAPI-labeled nuclei) in ventral (i.e NAc, Figure 1; One-way ANOVA, \(F_{(3,20)}=85.5; p<0.0001\), Veh vs THC, \(p<0.0001\)) and dorsal striatum (Figure 2, PLA signals/mm²: One-way ANOVA, \(F_{(3, 20)}=108.9, p<0.0001\); heteromer-expressing cells: One-way ANOVA, \(F_{(3,20)}=16.76; p<0.0001\)). The increase persisted after the 7-day withdrawal phase (WD+TAT-Sc) in both striatal subregions (Figures 1-2), but was abolished in animals treated with the TAT-D1 during this same withdrawal phase (Figures 1-2). 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 (Supp-Fig. 1).

Behavioral assessment of chronic THC administration and withdrawal

Due to the involvement of the D1-D2 heteromer in aversion, anxiety, addiction, and depression (65-67) we evaluated, according to the experimental timeline shown in Figure 3A, the effects of daily THC administration and withdrawal, on reward- or aversion- and anxiety-linked behaviors, and whether the D1-D2 would be associated with a specific behavior.

Sucrose test: During baseline assessment (T1), rats presented with a two-bottle choice of water and 1.5% sucrose showed a pronounced preference (88.6% ± 1.23 %) for the sucrose solution (Figure 3B1), which declined significantly after 9-day THC treatment (Figure 3B2; one-way ANOVA \(F_{(2,69)}=67.37; p<0.0001\); basal vs THC, \(q=16.17\), but was unchanged in 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<0.0001\] confirmed the THC-induced decrease in sucrose preference (Basal vs THC, \(p<0.0001\)); an effect countered by TAT-D1 during withdrawal (THC vs WD+TAT-D1, \(p<0.0001\)). In contrast, in the cohort that received the control-ineffective peptide (WD+TAT-Sc, Figure 3B4; repeated ANOVA \(F_{(2,17)}=6.99, p=0.001\]), the THC-induced decrease in sucrose consumption (Basal vs
THC, p=0.04), was not restored after the withdrawal phase [(THC vs WD+TAT-Sc, p=0.83) and (Basal vs WD+TAT-Sc, p=0.015)].

These results were confirmed by calculating the differences in sucrose consumption after the 9-day THC treatment (T2-T1) and 7-day withdrawal period (T3-T2) for each of the two groups receiving the peptides (WD+TAT-D1 and WD+TAT-Sc). Two-way ANOVA (Figure 3B5) revealed significant effect of treatment (F(1,20)=53.3; p<0.0001) and significant interaction between treatment and the peptide used (F(1,20)=17.9; p=0.0004). Tukey’s multiple comparisons test showed that chronic THC treatment had a similar decreasing effect in both groups (THC/WD+TAT-D1 versus THC/WD+TAT-Sc; p=0.19) and that TAT-D1 reversed the THC effect (THC/TAT-D1 vs WD+TAT-D1, p<0.0001), in contrast to TAT-Sc (THC/TAT-Sc vs WD+TAT-Sc, p=0.17).

These observations suggested that daily THC induced anhedonia-like behavior that persisted after 7-day drug withdrawal. 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 (72) (Figure 4; Supp-Figure 4). None of the parameters relative to exploratory or locomotor activities were significantly affected by the treatments. Basal locomotor activity (T1: 2609 ± 114 cm/5 minutes, Supp-Figure 4A), significantly decreased (Figure 4A and Supp-Figure 4A1, One-way ANOVA, F(2,66)=8.53; p=0.0005) after 9-day treatment with vehicle (Basal vs Veh, p=0.022) and THC (Basal vs THC, p=0.0009; Veh vs THC, p=0.68), possibly reflecting adaptation to the environment (from test-1:T1 to test-2:T2). Treatment with either the TAT-D1 or the control TAT-Sc peptides during the 7-day withdrawal phase had no significant effect on locomotion (Figure 4A and Supp-Figure 4A2).

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=0.92, Figure 4B, Panel B1); the number of entries (One-way ANOVA, F(4,24)=2.16; p=0.08, Figure 4B, Panel B2); the duration (One-way ANOVA, F(4,24)=1.42; p=2.4 Figure 4B, Panel B3), and the distance traveled in the center zone (One-way ANOVA, F(4,24)=1.85; p=0.13 Figure 4B, Panel B4).
**Dark/light chamber test:** In a test designed to detect anxiogenic/anxiolytic-like behavior, rats spent approximately 75% of the 5-minutes test period (T1) in the dark side of the chamber (187±5.4 sec; Figures 5A and 5B, basal), in accordance with the known preference of rodents for darkened environments. Rats treated daily with THC (T2) spent more time in the dark side of the chamber compared with their pre-drug treatment scores (Figures 5B, paired t-test with Welch's correction, p<0.0001, t=5.55, df=16). In contrast, vehicle-treated subjects showed no changes in dark/light preference (Figures 5A, paired t-test with Welch's correction, p=0.10, t=1.73, df=16). Indeed, in direct comparison to vehicle, 9-day THC treatment increased the time spent in the dark box (Figure 5C, unpaired t-test, p<0.0001, t=5.34, df=27.46).

In the subgroup treated with the control TAT-Sc peptide (Figure 5D), repeated measures ANOVA showed no significant effect \([F(2,17)=18.3, p=0.09]\) to reverse the THC-induced preference for the dark box (Basal vs THC, p=0.04), which persisted in spite of 7 days drug withdrawal (THC vs TAT-Sc, p=0.3). In contrast, TAT-D1 peptide (Figure 5E) significantly reversed (Repeated measures ANOVA, \([F(2,17)=18.3, p=0.003]\)) the THC-induced increase in time spent in the dark compartment (Basal vs THC, p=0.001, THC vs TAT-D1, p=0.005).

These observations were strengthened by another assessment of anxiogenic-like behavior, namely the number of times the rats emerged from the dark side (72). Repeated measures ANOVA applied to the WD+TAT-Sc group showed that treatment had an overall significant effect \([F(2,17)=9.6, p=0.008]\), with 9-day THC treatment decreasing the number of emergences (Basal vs THC, p=0.038, Figure 5F), which persisted after the 7-day withdrawal period in animals treated with the control peptide (THC vs TAT-Sc, p=0.28, Figure 5F). In contrast, repeated measures ANOVA \([F(2,17)=9.9, p=0.01]\) in the WD+TAT-D1 group (Figure 5G) showed that while THC had similar effect in lowering the number of emergences from the dark side (Basal vs THC, p=0.045, Figure 5G), treatment with TAT-D1 reversed the reduced emergence from the dark compartment (THC vs WD+TAT-D1, p=0.03, Figure 5G). As the number of increased passages of dark-to-light side is associated with improvement of anxiogenic-like behavior (72), 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 mesocortico-limbic neurons originating in the ventral tegmental area, including in the NAc (32-33; 51; 58). Since 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 in drug addiction were selectively targeted by WB analysis.

**CB1R activation:** As mentioned, CB1R mediates most THC effects. The expression of CB1R and its phosphorylation status were analyzed (Figure 6A). Compared with vehicle-treated animals, total CB1R expression was not affected (One-way ANOVA, F(4,24)=0.6; p=0.65) by repeated THC administration or after 7-days of drug withdrawal. However, the ratios of phosphorylated CB1R/total CB1R [pCB1R/CB1R] (Figure 6B, right panel) showed a significant effect of treatment (One-way ANOVA, F(4,24)=18.45; p=0.0001). The 9-day treatment with THC had a tendency (Veh vs THC, p=0.09) to increase the [pCB1R/CB1R] ratio, whereas, the 7-day withdrawal significantly increased the ratio in animals treated with the control peptide (Vehicle vs WD+TAT-Sc, p<0.0001; THC vs WD+TAT-Sc, p<0.009), which was attenuated by TAT-D1 treatment during withdrawal (Vehicle vs WD+TAT-D1, p=0.99; THC vs WD+TAT-D1, p=0.14). This suggests that daily treatment with THC tended to increase CB1R activation; an effect more pronounced after drug withdrawal and attenuated by D1-D2 disruption, linking thus D1-D2 to THC-CB1R and endocannabinoid system activation.

**Dopamine D1 and D2 receptors:** Besides the increase in D1-D2 heteromer (Figures 1-2), THC increased dopamine D1 receptor (D1R) expression in comparison to the vehicle group, (one-way ANOVA [F(4,24]=12.03; p<0.0001]; Figure 6B; Veh vs THC, p=0.0009), which remained high during withdrawal in animals treated with the control peptide (Vehicle vs WD+TAT-Sc, p<0.0001), but returned to vehicle-treated levels in animals treated with the TAT-D1 peptide (Vehicle vs WD+TAT-D1, p=0.04). D2R expression decreased after repeated THC treatment in comparison to vehicle (Figure 6B, right panel; One-way ANOVA, [F(4,24]=3.08; p=0.03], Veh vs THC, p=0.03), but returned to levels comparable to that in vehicle-treated animals after drug withdrawal (Veh vs WD+ TAT-Sc, p=0.68). TAT-D1 treatment during withdrawal maintained the reduction in D2R expression (Veh vs WD+TAT-D1: p=0.04).

**Modulation of signaling pathways in the NAc**
CaMKIIα: Calcium-mediated activation of CaMKIIα occurs through phosphorylation at Thr286 (pCaMKIIα) following D1-D2 heteromer activation (73-75) or following chronic THC treatment in nonhuman primate (69). Treatment had a significant effect on pCaMKIIα (One-way ANOVA, F(4,24)=92.8; p<0.0001; Figure 7A, left panel; Veh vs THC, p<0.0001). This effect was reversed after the 7-day withdrawal period whether rats received TAT-D1 (Veh vs WD+TAT-D1, p=0.98) or the control peptide (Veh vs WD+TAT-Sc, p=0.18).

TrkB: BDNF/TrkB signal activation is known to be involved in D1-D2 heteromer function (73), and is regulated following chronic THC administration in nonhuman primate (69). The 9-day treatment with THC and the 7-day withdrawal phase did not affect the total expression level of TrkB (Figure 7B, Left panel; F(4,24)=2.49; p=0.07). 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, F(4,24)=13.03; p<0.0001; Figure 7B, right panel), with daily THC treatment increasing this ratio (Veh vs THC, p=0.02) which subsequently decreased after withdrawal (THC vs WD+TAT-Sc, p<0.0001 and THC vs WD+TAT-D1, p=0.015).

cAMP-PKA-DARPP-32 pathway: The cAMP-PKA-DARPP-32 pathway, usually activated after acute drug intake (88), was silent, since no effect of treatment was observed in the phosphorylation of pERK (Figure 7C, left panel; One-way ANOVA, F(4,24)=1; p=0.42), in a target of cAMP-PKA activation, the AMPA receptor GluA1-subunit at Ser845 (pGluA-S845; Figure 7C, right panel; One-way ANOVA, F(4,24)=0.3; p=0.87). Although there was a significant effect of treatment on Thr-34-DARPP-32 phosphorylation (Figure 8A, left panel, One-way ANOVA, F(4,24)=15.03; p=0.0001), 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 in comparison to vehicle (p=0.001), to THC (p=0.004) and to the control group (WD+TAT-Sc, p<0.0001). Phosphorylation of DARPP-32 at Thr-75 (pT75-DARPP-32) was unchanged in all groups (Figure 8A, right panel; One-way ANOVA, [F(4,24)=2.17; p=0.10]).

Akt/GSK-3 pathway: Chronic (21 days) THC dephosphorylated (i.e. activated) GSK-3 in nonhuman primate (69). However, in rats the shorter period of treatment with THC (9-days) had no significant effect on GSK-3 (One-way ANOVA, [F(4,24)=1.02; p=0.42]; Figure 8B), nor on one of its modulators, Akt at Thr308 (F(4,24)=0.66; p=0.63) and Ser473 (F(4,24)=1.48; p=0.24).
Linking THC-induced effects to D1-D2 heteromer function and opioid Dyn/KOR action

Our results thus far suggest 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 postulated these effects may be mediated in part by the Dyn/KOR pathway. Overall, dynorphin expression was altered by treatment (One-way ANOVA, $F(4,24)=5.9; p=0.002$). Repeated THC administration significantly elevated dynorphin levels (Figure 9A; Veh vs THC, $p=0.06$), which persisted after the 7-day withdrawal period (THC vs WD+TAT-D1 $p=0.9$ and THC vs WD+TAT-TAT-Sc, $p=0.7$). THC treatment had no effect on total KOR expression (Figure 9B, left panel; One-way ANOVA, $F(4,24)=0.8; p=0.53$). However, analyzing the ratios of phosphorylated (pKOR) and total KOR [pKOR/KOR] (Figure 9B, right panel; One-way ANOVA $F(4,24)=16.2; p<0.0001$) showed that the 9-day treatment with THC increased the [pKOR/KOR] ratio significantly (vehicle vs THC, $p<0.0001$), which was restored to vehicle-treated values after withdrawal in both the TAT-D1 (vehicle vs WD+TAT=D1, $p=0.83$) and the TAT-Sc (Vehicle vs WD+TAT-Sc, $p=0.99$) groups.

To assess a probable direct link between D1-D2 heteromer activation and the Dyn/KOR pathway we proceeded as follows. Firstly, direct activation of D1-D2 by SKF-83959 administration (0.5 mg/kg s.c., 4 hrs) modified the dynorphin content in NAc (Figure 10A). Three dynorphin forms were documented on WB, pro-dynorphin species of molecular weights ~45 and ~25 kDa, and another of ~17 kDa, known as big dynorphin. Analysis showed that the pro-dynorphin species decreased significantly (ProDyn-45 kDa, $p=0.031$, t-test) or tended to decrease (ProDyn-25 kDa, $p=0.18$, t-test), whereas big-dynorphin, more commonly known to reflect released dynorphin, was significantly increased after SKF-83959 treatment ($p=0.006$, t-test). Due to its aversive and pro-anxiogenic-like effects (66), SKF-83959 led to increased self-grooming (Figure 10B, One-way ANOVA, $F(3,20)=27.7; p<0.0001$); an effect due to D1-D2 heteromer activation, since the TAT-D1 peptide, but not the control peptide, was able to inhibit the SKF-83959-induced grooming. Interestingly, pre-treatment with the KOR antagonist, nor-Binaltorphimine (nor-BNI, 10 mg/kg, i.p. 24 h), inhibited the SKF-83959-induced and D1-D2 heteromer-mediated grooming (Figure 10C, One-way ANOVA, $F(2,18)=38.2, p<0.0001$). Collectively, these data suggest 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 three neurotransmitter systems, dopamine, cannabinoid, and opioid occurred at the molecular level. In contrast to the unmodified CB1 receptor 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 (eCB) system. Similar to many GPCRs, phosphorylated CB1R may undergo desensitization, internalization, and/or down-regulation following chronic exposure to cannabinoids (81-84). In humans, CB1R down-regulation was shown to be correlated with duration of cannabis smoking and was reversible after ~4 weeks of cannabis abstinence (85), but was regionally specific, with significant downregulation occurring selectively in cortical brain regions, but not observed in the caudate, putamen or ventral striatum (NAc) (85). This later result is in line with the present data showing no change in total CB1R expression in 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, linking thus 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 NAc. Reduced D2R availability was reported in human misusers of stimulants, alcohol, cocaine and in long-time marijuana users (86-87; 8-9). Thus, recurrent exposure to drugs that overstimulate dopamine signaling may lead to decreased D2R in line with the decrease in D2R-mRNA (69) or D2R-protein (present study) after chronic THC.

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 non-human 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 (PPI) inhibition (88). This suggests that increased heteromer density may function initially to decrease this super-activated reward pathway as was shown in a cocaine-administration model (66). However, prolonged/repeated D1-D2 activation induces aversion and anhedonia due to 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 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 CaMKIIα activation, and BDNF/TrkB signaling, both of which are part of the well-documented D1-D2-linked calcium signal (73); an effect similar to that elicited by chronic THC in adult rhesus monkey (69), which indicates that repeated THC may activate, in part, calcium-CaMKIIα and BDNF/TrkB signaling through increased D1-D2 heteromer expression/activation. Elevated BDNF-TrkB activity in NAc contributes to depressive-anhedonic behaviors in rats (89), and was observed after escalating marijuana use among adolescents and also in adults with cannabis use disorder (90-91), with dynorphin being proposed as a downstream BDNF effector in the striatum (90-93). Intriguingly, increased dynorphin expression and enhanced phosphorylation of its receptor KOR, were observed following THC repeated treatment and withdrawal, adding thus another layer of interaction between the three 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 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-BNI, 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 NAc to cannabinoid, dopamine, and opioid signaling cascades (Figure 11). According to literature (94-96), a single exposure to THC activates CB1R to inhibit the GABAergic input, leading to increased dopamine release (Figure 11, step 2). Repeated THC exposure, however, elevates D1-D2 heteromer density (Figure 11, step 3), which is sustained after spontaneous withdrawal for 7 days. Dopamine activity at the D1-D2 heteromer would activate the well-known (73) Gq-mediated increased calcium mobilization and activation of calcium-linked signaling cascades (Figure 11, step 4) including increased CaMKII activity and BDNF expression (Figure 11, step 5). In analogy with the biochemical cascade triggered by cocaine action (97-98), BDNF/TrkB activation would lead to increased CREB activation, prodynorphin synthesis and processing (Figure 11, step 6). Alternatively, BDNF can activate TrkB on all MSNs types (99-100; 66), resulting in increased dynorphin release from D1-MSNs and D1/D2-MSNs (Figure 11, step 6). Dynorphin would activate its receptor, KOR (101), present on presynaptic dopamine neurons (Figure 11, step 7). This would lead to decreased dopamine release in NAc after chronic drug treatment (Figure 11, step 8), which would contribute to the anhedonia and anxiogenic-like behavior observed in this study (Figure 11, step 9) and to the general aversion-like effect after chronic drug treatment and repetitive activation of the heteromer D1-D2 (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 male adult rats only. We anticipate there would be many differences due to sex and other factors such as age in the cannabinoid, dopaminergic and opioidergic systems. Especially, significant sex (102) and age (75) differences in D1-D2 expression in NAc were documented, and ongoing studies are examining the effects of THC in these populations.

One interesting result in the present study is that disrupting D1-D2 heteromer activity during withdrawal fostered remission from the observed anhedonia and anxiogenic 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 47-95% of heavy users (103-106). As withdrawal symptoms in human cannabis users are a catalyst for ongoing drug seeking and relapse, this novel strategy should be further evaluated for providing symptom relief and a stabilizing affect to remain in treatment for cannabis use disorder.

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 (present 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 SUDs, but conceivably to also prevent relapse, targeting the enormous unmet needs of compulsive motivational addiction states for which currently there are no effective treatments.
Acknowledgements:

**Funding:** This project was funded by grants from NIDA DA042178 (to BKM and SRG), CIHR PJT-148633 (to SRG).

**Author contributions:** AH, BKM, and SRG conceived and designed the experiments; AH performed the experiments and analyzed the results; AH and SRG wrote the manuscript and BKM, AH, and SRG edited the manuscript.

**Conflict of Interest:** The authors report no biomedical financial interests or potential conflicts of interest.
Figure Legends

**Figure 1. Evaluation of D1-D2 heteromer expression in NAc.**
Rats received chronic treatment (daily i.p. injection for 9 days) with vehicle or THC (1mg/kg). Groups of rats went through drug withdrawal (WD) for 7 days, during which they received daily administration of intranasal TAT-D1 peptide (WD+TAT-D1) or a control TAT-scrambled peptide TAT-Sc (15 µg). Sections from striatum were evaluated for expression of the dopamine D1-D2 heteromer using *in situ* Proximity Ligation Assay (PLA) as described in Materials and Methods. (A) Representative images and magnifications depicting the PLA signal (red dots) together with cell nuclei identification (by DAPI) for each group. (B) Bar graphs representing the analysis of the number of cells expressing D1-D2 heteromer depicting averaged values from each rat (left) and analysis of density of D1-D2 PLA signals (dots) per mm² (right). Data are means ± SEM. (N=6 rats/condition, 3-4 sections from each rat). Scale bars are 10 µm. One-way ANOVAs with multiple comparisons: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. ns: not significant (p>0.05).

**Figure 2. Evaluation of D1-D2 heteromer expression in CPu.**
Rats received chronic treatment (daily i.p. injection for 9 days) with vehicle or THC (1mg/kg). Groups of rats went through a withdrawal period (WD) for 7 days, during which they received daily intranasal administration of TAT-D1 peptide (WD+TAT-D1) or a control TAT-scrambled peptide (WD+TAT-Sc). Sections from the striata were evaluated for expression of the dopamine D1-D2 heteromer using *in situ* PLA as described in Materials and Methods. (A) Representative images and magnifications depicting the PLA signal (red dots) and cell nuclei (DAPI) for each group. (B) Bar graphs representing the analysis of the number of cells expressing the D1-D2 heteromer from each rat (left) and analysis of density of D1-D2 PLA signals (dots) per mm² (right). Data are means ± SEM. (N=6 rats/condition, 3-4 sections from each rat). Scale bars are 10 µm. One-way ANOVAs with multiple comparisons: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. ns: not significant (p>0.05).

**Figure 3. Evaluation of hedonic/anhedonic-like behavior using the sucrose preference test.**
A. **Experimental design.** Rats underwent a basal behavioral assessment (T1) after one-week of handling and were randomly assigned to receive daily intraperitoneal (i.p.) injections of either THC (1 mg/kg) or a control/vehicle solution for a total of nine injections. Rats underwent a second behavioral assessment (T2) 24 hours after the last injection of THC (n=18) or vehicle (n=18) and six rats of each group were sacrificed (Vehicle, THC). The remaining animals underwent spontaneous drug withdrawal over seven days, during which they received daily intranasal insufflation (15 µg) of either the D1-D2 selective interfering peptide, TAT-D1 (WD+TAT-D1), or its control scrambled control peptide, TAT-Sc (WD+TAT-Sc). These rats were subjected to another round of behavioral tests (T3) 24 hours after the last intranasal administration of the peptides and then sacrificed.

B. **Sucrose Preference Test.** Rats were presented with a two-bottle choice of sucrose (1.5%) or water. In (B1) the average preference of all rats at the basal level is shown. (B2) Data representing
the preference following treatment with vehicle or THC for 9 days (One-way ANOVA). The results of the sucrose preference test for the withdrawal groups that received TAT-D1 or TAT-scrambled peptides are presented in (B3) and (B4), respectively (Repeated measures ANOVA). In (B5) data recapitulating the differences in sucrose consumption 24 hrs (T2-T1) and 7 days (T3-T2) after cessation of THC treatment in the groups that received intranasal TAT-D1 or the control peptide TAT-Sc (Two-way ANOVA). N=6 rats per condition *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, ns: not significant (p>0.05). Error bars indicate SEM.

**Figure 4. Open field test.**
Locomotor activity was monitored using the open-field for rats treated chronically (daily i.p. injection for 9 days) with vehicle (veh) or THC (1mg/kg), and after withdrawal from THC for 7 days, during which they received daily intranasal TAT-D1 peptide or control TAT-scrambled peptide (15 µg). The different measures included (A) the distance traveled, and (B): latency to enter (B1), number of entries (B2), time spent (B3) and distance traveled (B4) in the center compartment during the open-field test. N=6 rats per condition, one-way ANOVAs; **p≤0.01, ns: not significant (p>0.05). Error bars indicate SEM. Basal refers to activity of all subjects prior to initiation of experimental procedures. Veh refers to control animals treated for 9 days with the vehicle for THC. THC is the cohort treated with THC for 9 days. WD+TAT-Sc are animals treated with THC for 9 days and then subject to THC withdrawal for 7 days, during which they are treated daily with scrambled peptide intranasally. WD+TAT-D1 are animals treated with THC for 9 days and then subject to THC withdrawal for 7 days, during which they are treated daily with TAT-D1 peptide intranasally.

**Figure 5. Light/dark box anxiety test.**
Effects of chronic treatment (daily i.p. injection for 9 days) with vehicle (A) or THC (1mg/kg, B) on the time spent in the dark compartment (t-tests, **p≤0.01, ns: not significant (p>0.05). (C) Comparison of the effects of Vehicle versus THC treatments on the time spent in the dark side (t-test, ***p≤0.001). Error bars indicate SEM. (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 ANOVAs, *p≤0.05, **p≤0.01, ns: not significant (p>0.05). (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 ANOVAs, *p≤0.05, **p≤0.01, ns: not significant (p>0.05). N=6 rats per condition. Error bars indicate SEM. Basal refers to activity of all subjects prior to initiation of experimental procedures.

**Figure 6. THC-induced changes in D1 and D2 dopamine receptors and the CB1 receptor.**
Western blot analysis of homogenates from rats received chronic treatment (daily i.p. injection for 9 days) with vehicle or THC (1mg/kg). Groups of rats went through a withdrawal period (WD) for 7 days, during which they received daily intranasal TAT-D1 (WD+TAT-D1) peptide or a control
TAT-scrambled peptide (WD+TAT-Sc). (A) Evaluation of the total expression and phosphorylation (pCB1R) of the CB1 receptor and their ratio (pCB1R/total CB1R) in NAc. (B) Evaluation of the expression of dopamine D1 and D2 receptors in NAc. Data are means ± SEM. N=6 rats per group. One-way ANOVAs with multiple comparisons: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, ns: not significant (p>0.05).

**Figure 7. THC-induced neuroadaptive changes in signaling proteins: pCaMKII and pTrkB.**
Western blot analysis of homogenates from rats that received chronic treatment (daily i.p. injection for 9 days) with vehicle or THC (1mg/kg). Groups of rats went through drug withdrawal (WD) for 7 days, during which they received daily intranasal TAT-D1 (WD+TAT-D1) peptide or a control TAT-scrambled 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/total TrkB [pTrkB/TrkB] (right panel). Data are means ± SEM. N=6 rats per group. One-way ANOVAs with multiple comparisons: *p≤0.05, ****p≤0.0001, ns: not significant (p>0.05).

**Figure 8. THC-induced changes in signaling proteins: DARPP-32, GSK3 and Akt.**
Western blot analysis of homogenates from rats that received chronic treatment (daily i.p. injection for 9 days) with vehicle or THC (1mg/kg). Groups of rats went through drug withdrawal (WD) for 7 days, during which they received daily intranasal TAT-D1 (WD+TAT-D1) peptide or control TAT-scrambled peptide (WD+TAT-Sc). (A) Analysis of the phosphorylation of DARPP-32 at Thr-34 (pT34) and Thr-75 (pT75) sites in the different groups relative to GAPDH. (B-C) Evaluation of phosphorylation of (B) Akt phosphorylation at two sites (Thr-308 and Ser-473) and (C) GSK3 (pGSK3). Data are means ± SEM. N=6 rats per group. One-way ANOVAs with multiple comparisons: **p≤0.01, ****p≤0.0001, ns: not significant (p>0.05).

**Figure 9. THC-induced changes in opioid peptides and the kappa opioid receptor.**
Western blot analysis of homogenates from rats that received chronic treatment (daily i.p. injection for 9 days) with vehicle or THC (1mg/kg). Groups of rats went through drug withdrawal (WD) for 7 days, during which they received daily intranasal TAT-D1 (WD+TAT-D1) peptide or control TAT-scrambled peptide (WD+TAT-Sc). (A) Analysis of the expression of dynorphin (Dyn) in the different groups in comparison to GAPDH. A form of 17-18 kDa, representing big-dynorphin, usually called dynorphin (Dyn) is shown. (B) Analysis of the total expression and phosphorylation of kappa opioid receptor (KOR) in the different groups. Data are means ± SEM. N=6 rats per group. One-way ANOVAs with multiple comparisons: *p≤0.05, **p≤0.01, ****p≤0.0001, ns: not significant (p>0.05).

**Figure 10. Link between THC-induced signaling cascades, dynorphin/KOR activation and dopamine D1-D2 heteromer function.**
(A) Analysis of the levels of prodynorphin-derived peptides (45, 25 and 17 kDa) in NAc of rats treated with SKF 83959 (0.5 mg/kg, s.c.) or vehicle for 4 hours. WB reveals two isoforms of prodynorphin, 48 and 25 kDa, and one form of 17-18 kDa, representing big-dynorphin, usually called dynorphin (Dyn). Analyses of each band intensity relative to GAPDH are summarized. Data are means ± SEM. N=3 rats per group. *p≤0.05, **p≤0.01, ***p≤0.001, ns: not significant (p>0.05). In (B) self-grooming behavior was analyzed in rats treated with SKF 83959 (0.5 mg/kg s.c., 4 hours prior) after intranasal pre-treatment 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, i.p., 24 hrs prior). Data are means ± SEM. N=6 rats per group. One-way ANOVAs with multiple comparisons: *p≤0.05, **p≤0.01, ***p≤0.001, ns: not significant (p>0.05).

Figure 11. Postulated mechanism of D1-D2 heteromer action during chronic THC and withdrawal.

Acute THC activates presynaptic CB1R (1), which in turn inhibits GABA signaling resulting in increased dopamine release (2). Chronic THC induces an increase in D1-D2 heteromer numbers (3), which is sustained or heightened during drug withdrawal. Activation of the D1-D2 heteromer by dopamine leads to increased intracellular calcium mobilization and activation of calcium-mediated signaling (4), leading to increased BDNF. By activating TrkB present on D1-MSNs (5) and activating the synthesis and processing of prodynorphin within D1-D2 heteromer-expressing neurons, BDNF would lead to increased levels and release of dynorphin (6). Dynorphin would activate kappa opioid receptor (7) on presynaptic dopamine neuron terminals, leading to decreased dopamine release and reduced synaptic levels of dopamine (8). This reduction in NAc dopamine levels would be responsible for anhedonia and anxiogenic-like behavior observed in this study and to the general aversion-like effect observed after repetitive activation of the heteromer D1-D2 (9).
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Figure 1

A. **D1-D2-PLA/DAPI in the NAc**

![Images showing D1-D2-PLA/DAPI in Vehicle, THC, WD+TAT-Sc, and WD+TAT-D1 conditions.]

B. 

**Percent of total cells (DAPI):**

- Vehicle
- THC
- WD+TAT-Sc
- WD+TAT-D1

**D1-D2 PLA dots / mm^2:**

- Vehicle
- THC
- WD+TAT-Sc
- WD+TAT-D1
Figure 2

A. D1-D2 PLA/DAPI in the CPu

B. Percent of total cells (DAPI)

Vehicle

THC

WD+TAT-Sc

WD+TAT-D1

Percent of total cells (DAPI)

Vehicle

THC

WD+TAT-Sc

WD+TAT-D1

100
80
60
40
20
0

10000
20000
30000
D1-D2 PLA dots / mm²

Vehicle

THC

WD+TAT-Sc

WD+TAT-D1

Percent of total cells (DAPI)

Vehicle

THC

WD+TAT-Sc

WD+TAT-D1

10000
20000
30000
D1-D2 PLA dots / mm²
A. Experimental design

Handling (5 days)

One daily i.p. injection of THC (1mg/kg) or vehicle for 9 days

Behavioral tests
Basal

One daily Intranasal delivery of TAT-D1 or TAT-Sc peptides (15 µg) for 6 days

Behavioral tests
Effects of THC

Behavioral tests
Effects of peptides on withdrawal

Brain collection
Groups:
• Veh
• THC

B. Sucrose preference test

B1.
Sucrose preference (%)

Basal (T1) all rats

B2.
Sucrose preference (%)

Basal (T1) THC (T2) Vehicle (T2)

B3.
Sucrose preference (%)

Basal (T1) THC (T2) WD+ TAT-D1 (T3)

B4.
Sucrose preference (%)

Basal (T1) THC (T2) WD+ TAT-Sc (T3)

B5.
Variation in sucrose consumption (%)

THC (T2-T1) WD (T3-T2)
**Figure 4** Open Field test

A. Locomotor activity

B. Center zone parameters

B1. Latency-Center zone

B2. Entries-Center zone

B3. Duration-Center zone

B4. Distance in the center zone
**Figure 5  Dark/Light box**

A. **Vehicle effect**

B. **THC effect**

C. **Vehicle versus THC**

D. **TAT-Sc**

E. **TAT-D1**

F. **TAT-Sc**

G. **TAT-D1**
Figure 6

A. CB1R/GAPDH

B. D1R/GAPDH

D2R/GAPDH

Veh THC WD+TAT-Sc WD+TAT-D1

Veh THC WD+TAT-Sc WD+TAT-D1

Veh THC WD+TAT-Sc WD+TAT-D1

Veh THC WD+TAT-Sc WD+TAT-D1

pCB1R/CB1R/GAPDH (% of Vehicle)

pD1R/CB1R/GAPDH (% of Vehicle)

pD2R/CB1R/GAPDH (% of Vehicle)

*p = 0.09

**p < 0.01

***p < 0.001

****p < 0.0001

ns = not significant
Figure 7

A. pCaMKIIα/GAPDH

B. TrkB/GAPDH

C. pERK/GAPDH

D. pGluA-S845/GAPDH

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Figure 8

A. pT34-DARPP-32/GAPDH

B. pAkt-308/GAPDH

C. pGSK-3/GAPDH
Figure 10

A. MW (kDa) 

ProDyn~45 
GAPDH 
ProDyn~25 
Big Dyn~17

B. ProDyn-45 kDa 

C. ProDyn-25 kDa 

Big Dyn-17 kDa

Grooming behavior (seconds)
Figure 11

Acute THC

Increased activity

Chronic THC Withdrawal

Increased DA release

Decreased DA release

DA neurons

CB1R

Glutamate

D2R

D1R

TrkB

KOR

DA

THC

GABA

Increased activity

BDNF

Dynorphin

Anhedonia

Anxiogenic-like Aversion

Acute THC

Decreased DA release

BDNF

D1-D2

Ca^{2+}

D1-MSN

D2-MSN

TrkB

TrkB

GABA

DA neurons

Increased activity

BDNF

Dynorphin

Anhedonia

Anxiogenic-like Aversion