

Cannabidiol Produces Distinct U-Shaped Dose-Response Effects on Cocaine-Induced Conditioned Place Preference and Associated Recruitment of Prelimbic Neurons in Male Rats

Hermína Nedelescu, Grant E. Wagner, Genna L. De Ness, Ayla Carroll, Tony M. Kerr, Jingjun Wang, Saiwen Zhang, Stephen Chang, Amy H. Than, Nora E. Emerson, Nobuyoshi Suto, and Friedbert Weiss

ABSTRACT

BACKGROUND: Cannabidiol (CBD) has received attention for the treatment of substance use disorders. In preclinical models of relapse, CBD attenuates drug seeking across several drugs of abuse, including cocaine. However, in these models CBD has not been consistently effective. This inconsistency in CBD effects may be related to presently insufficient information on the full spectrum of CBD dose effects on drug-related behaviors.

METHODS: We address this issue by establishing a full dose-response profile of CBD's actions using expression of cocaine-induced conditioned place preference as a model for drug-motivated behavior in male rats and by concurrently identifying dose-dependent effects of CBD on underlying neuronal activation and distinct neuronal phenotypes showing dose-dependent activation changes. Additionally, we established CBD levels in plasma and brain samples.

RESULTS: CBD produced linear increases in CBD brain/plasma concentrations but suppressed conditioned place preference in a distinct U-shaped manner. In parallel with its behavioral effects, CBD produced U-shaped suppressant effects on neuronal activation in the prelimbic but not infralimbic cortex or nucleus accumbens core and shell. RNAscope in situ hybridization identified suppression of glutamatergic and GABAergic (gamma-aminobutyric acidergic) signaling in the prelimbic cortex as a possible cellular mechanism for the attenuation of cocaine-induced conditioned place preference by CBD.

CONCLUSIONS: The findings extend previous evidence on the potential of CBD in preventing drug-motivated behavior. However, CBD's dose-response profile may have important dosing implications for future clinical applications and may contribute to the understanding of discrepant CBD effects on drug seeking reported in the literature.

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Cannabidiol (CBD), the main nonpsychoactive and nonaddictive constituent of *Cannabis sativa*, has received significant interest as a therapeutic agent for the treatment of substance use disorders (SUDs). Growing evidence suggests that CBD ameliorates susceptibility to stress and craving induced by drug contexts, factors that play a prominent role in the process of drug-motivated behavior. In reinstatement models of relapse, CBD attenuated heroin, cocaine, and alcohol seeking induced by drug-associated stimuli or stress (1,2). Similarly, CBD attenuated renewal of cocaine, morphine, and methamphetamine seeking in conditioned place preference (CPP) models of relapse (3–5). Therefore, CBD may have pharmacotherapeutic promise for treating drug addiction. Strengthening this hypothesis are clinical findings suggesting that CBD reduces cue-induced craving and anxiety in abstinent heroin users (6).

Despite the promising implications of these findings, investigators in a considerable number of studies failed to observe positive effects of CBD on drug-seeking behaviors [reviewed in Chye *et al.* (7)]. The potential for CBD to treat SUDs, therefore, requires improved understanding. Contributing to the discrepant findings in the literature is the fact that previous studies on CBD's anti-addiction potential typically employed 1- or 2-dose designs such that uncertainty regarding the effective CBD dose range remains (7,8). We have recently reported that a single dose of CBD attenuated context- and stress-induced reinstatement of cocaine and alcohol seeking with stable effects across 7 days of treatment and testing. However, maximal attenuation of reinstatement occurred well before CBD levels in brain tissue and plasma reached their peak (1), raising questions about the dose dependence of CBD's effects on drug seeking. Indeed, in the

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limited number of studies that have employed multidose designs to investigate effects on drug seeking in the CPP model, CBD was effective at intermediate but not high or low doses (3,9). Similar results were obtained for CBD effects on conditioned fear (10), and a U-shaped dose-response profile has been reported for attenuation of experimental anxiety by CBD (11) [but see Gonzales-Cuevas *et al.* (1)]. Therefore, the dose-response profile of CBD on drug-motivated behavior remains to be more systematically characterized. U-shaped dose-response effects will have important implications for the presumptive potential of CBD in treating SUDs and for the understanding of the divergent effects of CBD on drug seeking reported in the literature.

This study, therefore, was designed to establish a comprehensive dose-response profile of CBD's actions, using expression of cocaine-induced CPP as a model for drug-motivated behavior and, in parallel, to address the expectation that behaviorally relevant pharmacological actions of CBD must be reflected by corresponding dose-dependent effects on underlying brain function. Little is known, to date, about the neurobiological mechanisms through which CBD modifies drug-seeking behavior (2,3,12). However, the medial prefrontal cortex and nucleus accumbens (NAc)—key components of the corticostriatal circuitry known to regulate drug seeking (13–15)—have been implicated in mediating several of CBD's behavioral effects (16,17). We therefore targeted subregions of the medial prefrontal cortex and NAc to establish CBD's effects on CPP-associated neuronal activation and to identify the phenotypes of neurons showing CBD-induced changes in neuronal activity, using RNAscope *in situ* hybridization.

METHODS AND MATERIALS

Animals

Male Wistar rats (Charles River Laboratories) weighing 300 to 350 grams at time of arrival were used. Rats were housed in a vivarium controlled for temperature (21 ± 2 °C) and humidity on a reverse 12-hour light/dark cycle, with *ad libitum* availability of food and water. Training and testing sessions took place during the dark phase. All experimental procedures were conducted in accordance with the National Institutes of Health's *Guidelines for the Care and Use of Laboratory Animals* (18) and approved by the Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrochloride (National Institutes of Health) was dissolved in sterile physiological saline to a concentration of 10 mg/mL. CBD was administered transdermally in a gel preparation (Zynerba Pharmaceuticals, Inc.) as previously reported (1). The transdermal route was chosen with translational relevance in mind. Oral CBD is associated with low (~6%) bioavailability (19) and potential for conversion into psychoactive cannabinoids in gastric fluid (20). Transdermal administration avoids these limitations and produces stable and sustained plasma CBD levels (21,22). CBD or vehicle gel was applied to shaved skin on the back/neck region and rats were single housed for 4 hours before behavioral testing.

Experimental Design and Procedures

The behavioral training and testing sequence consisted of 5 phases: 1) Habituation to daily gel application for all rats (vehicle gel; 7 days). 2) Compartment bias testing: A biased place-conditioning procedure was used. Compartment bias was established in 30-minute daily sessions with free-roaming access to both compartments for 7 days. The preferred versus nonpreferred side was determined for each rat from the average of time spent in each compartment across the last 3 days of this phase. Rats then were assigned to treatment with 1 of 5 CBD doses or vehicle. 3) Place conditioning for 10 days by pairing 10 mg/kg of cocaine with the nonpreferred compartment and pairing saline with the preferred compartment for 30 minutes daily, alternating between cocaine and saline days. Both cocaine and saline were administered intraperitoneally immediately before placement into the respective compartment. 4) CBD treatment: Following the final conditioning session, rats were treated once daily with 1 of 5 CBD doses (2.5, 5, 7.5, 10, or 15.0 mg/kg) or vehicle for 4 days. 5) Twelve hours after the final treatment, expression of CPP was determined by measuring the total amount of time spent by each animal in the cocaine-paired compartment during 30-minute free-roaming sessions (without receiving cocaine or saline). A naïve group in which rats were sacrificed from home cage and a control group in which rats received CBD vehicle and were exposed to the whole experiment except the CPP test were both included for histological investigations.

Plasma and Brain Tissue CBD Levels

CBD concentrations in plasma and brain tissue were measured in all rats 12 hours after the final CBD treatment. Animals were deeply anesthetized and euthanized to harvest brain tissue and trunk blood for analysis. Blood samples were centrifuged at 10,000 rpm for 10 minutes and the resulting plasma separated and stored at -80 °C until analyzed. Brains were flash frozen in isopentane and stored at -80 °C until analyzed.

Histology

Ninety minutes from the start of the CPP test [i.e., when Fos protein expression is known to reach peak (23)], rats were deeply anesthetized and transcardially perfused with 4% paraformaldehyde for immunohistochemistry or decapitated before harvesting fresh brains to flash freeze for RNAscope *in situ* hybridization experiments. Brains were processed according to standard immunohistochemical procedures as previously described (24). A rabbit monoclonal c-Fos antibody (catalog number 2250; Cell Signaling Technology) was used at a dilution of 1:8000 and DAB to visualize Fos-expressing activated neurons. RNAscope protocols (Advanced Cell Diagnostics) were used to prepare flash-frozen tissue for *in situ* hybridization using custom-made antisense messenger RNA (mRNA) probes. The mRNA probes c-Fos (activated neurons), Slc17a7 (glutamatergic neurons), Slc32a1 (GABAergic [gamma-aminobutyric acidergic] and glycinergic neurons), and Chat (cholinergic neurons) were used to identify the specific phenotype of activated neurons.

Data Analysis

CBD plasma and brain concentrations were analyzed by Pearson's r to determine the linear fit between CBD doses and resulting CBD levels. Behavioral, Fos protein, and *c-Fos* mRNA population data were non-normally distributed; therefore, nonparametric tests were employed. CPP was measured by comparing postconditioning scores (i.e., time spent in each compartment during the CPP test) to the respective pre-conditioning scores (i.e., time spent in each compartment during baseline preference, before CBD treatment). Differences between pre- and postconditioning scores then were analyzed by nonparametric Friedman analysis of variance and pairwise comparisons with Bonferroni correction to identify the degree of CPP relative to preconditioning behavior for each CBD dose group. Effect-size estimates for the Friedman test (referred to as W) were calculated as follows: $W = \chi^2/n(k - 1)$, where χ^2 is the Friedman test statistic value, n is the total number of rats, and k is the number of measurements per rat. Dose-related differences in CPP performance were analyzed by comparing test score distributions using pairwise Kolmogorov-Smirnov tests. A percent of vehicle index was calculated by dividing the time spent on test day in the drug compartment for each rat by the vehicle group's average time spent in the drug compartment, obtaining a percent of vehicle time index, which was statistically analyzed via the Kruskal-Wallis test followed by pairwise comparisons with Bonferroni correction. The time spent in the saline and cocaine compartments for each CBD dose group (see [CPP compartment preference before and after conditioning](#) in the Supplement) (Figure S1) was analyzed by Kruskal-Wallis test and pairwise comparisons with Bonferroni corrections. Similarly, locomotor activity during the 30-minute CPP tests (see [Locomotor activity](#) in the Supplement) (Figure S2) was analyzed for statistical differences by the Kruskal-Wallis test. Differences in Fos protein expression were analyzed by Kruskal-Wallis tests and pairwise comparisons. Effect-size estimates η^2 for the Kruskal-Wallis test were calculated as follows: $\eta^2_H = (H - k + 1)/(n - k)$, where H is the value obtained from the Kruskal-Wallis test, k the number of groups, and n the total number of rats. RNA-scope data were analyzed by Mann-Whitney U test for differences in mRNA expression levels between effects of vehicle ($n = 4$) and the behaviorally most effective CBD ($n = 5$) dose.

RESULTS

Brain and Plasma CBD Concentrations

The data revealed a significant linear dose-response relationship between transdermal CBD doses and both CBD brain and plasma levels ($n = 6$ per group). A highly significant positive correlation ($r_6 = 0.98$, $p < .001$) was found between CBD concentrations in homogenized brain tissue and the 5 CBD doses (Figure 1A). Similarly, CBD levels in plasma were significantly ($r_6 = 0.99$, $p < .001$) positively correlated with CBD doses (Figure 1B).

CBD Reduces CPP With a U-Shaped Dose-Response Profile

The CPP procedure (Figure 2A) was based on a biased design; however, approximately 50% of the rats failed to show

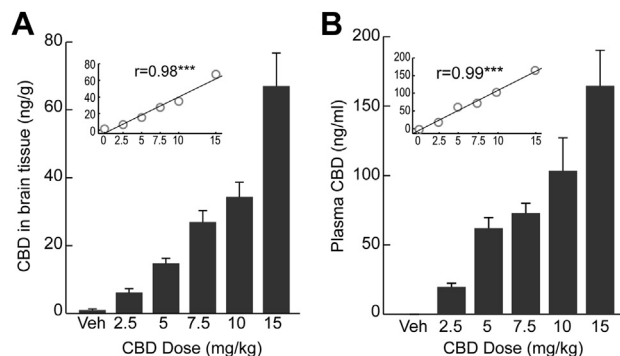


Figure 1. Mean \pm SEM CBD concentrations in (A) brain tissue and (B) plasma produced by each of the 5 transdermal CBD doses. (Insets) A highly significant positive linear relationship ($p < .001$) was recorded between CBD doses and corresponding CBD levels in both (A) brain and (B) plasma. *** $p < .001$. CBD, cannabidiol; Veh, vehicle.

significant compartment bias. These animals were, therefore, randomly assigned the drug or vehicle compartment. During the CPP test, time spent in the cocaine-paired compartment was significantly increased compared with preconditioning preference (vehicle, $n = 19$) and remained increased for rats treated with the 2.5-mg/kg ($n = 14$) and 15-mg/kg ($n = 14$) CBD doses ($p < .05$), but not the 5-mg/kg ($n = 14$), 7.5-mg/kg ($n = 8$), or 10-mg/kg ($n = 5$) doses (Friedman: $\chi^2_1 = 38.37$, $p < .001$ and post hoc Wilcoxon signed-rank analysis with Bonferroni adjustments) (Figure 2B). Thus, the intermediate CBD doses (5, 7.5, and 10 mg/kg) were statistically effective in diminishing cocaine CPP, whereas the low (2.5 mg/kg) and high (15 mg/kg) doses were not (Figure 2B). The overall effect size of CBD on the time spent in the cocaine-paired compartment was 0.50 ($W = 0.50$, effect-size estimate for the Friedman test). Comparisons among dose groups revealed that only the 7.5 mg/kg dose reduced CPP scores relative to vehicle-treated rats (pairwise Kolmogorov-Smirnov test, $p < .01$) (Figure 2B). Similarly, analysis of the percent of vehicle preference across treatment groups confirmed 7.5 mg/kg CBD as the only statistically effective dose in suppressing cocaine CPP (Kruskal-Wallis, $H_5 = 16.26$, $p < .01$; post hoc pairwise comparison with Bonferroni correction, $p < .05$) (Figure 2C). Locomotor activity, as a measure of nonspecific psychomotor activation or inhibition by CBD, did not differ statistically between vehicle control rats and CBD-treated rats (Kruskal-Wallis, $H_5 = 2.69$; not significant) (Figure S2).

CBD Reduces CPP-Associated Neuronal Activation in the Prelimbic Cortex With a U-Shaped Dose-Response Profile

To establish the extent to which the behaviorally relevant pharmacological actions of CBD dose-dependently modify presumptive underlying brain function, the effects of the behaviorally effective 7.5-mg/kg CBD dose on CPP-associated neuronal activation were contrasted with those of the behaviorally noneffective doses at both the high (15 mg/kg) and low (2.5 mg/kg) end. Four key neuroanatomical areas forming the corticostriatal circuitry were targeted for this purpose: the infralimbic cortex (IL), prelimbic cortex (PL),

U-Shaped Cannabidiol Dose-Response Profile

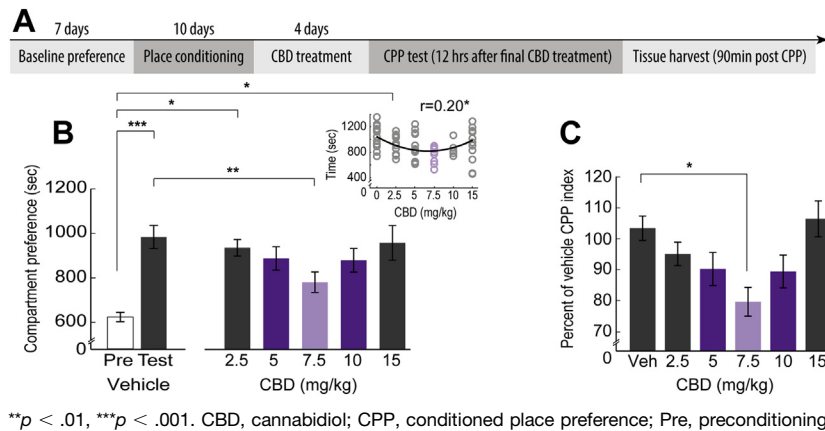


Figure 2. Effects of CBD on cocaine-induced CPP. **(A)** Sequence of experimental procedures. **(B)** Vehicle: In vehicle controls, mean \pm SEM post-conditioning scores (Test) were significantly higher than corresponding preconditioning scores ($p < .001$). CBD effects: CPP scores were significantly reduced by the 7.5-mg/kg CBD dose ($p < .01$ vs. vehicle test). CBD also reduced cocaine-induced CPP at the adjacent 5-mg/kg and 10-mg/kg doses; chamber preference at these doses was not statistically different from the preconditioning scores. (Inset) CBD dose-response function: Effects of CBD doses on CPP followed a significant quadratic relationship ($p < .05$). **(C)** Cocaine-induced CPP, expressed as mean \pm SEM percent of vehicle performance, was significantly reduced by the 7.5-mg/kg CBD dose ($p < .05$ vs. vehicle). * $p < .05$, ** $p < .01$, *** $p < .001$. CBD, cannabidiol; CPP, conditioned place preference; Pre, preconditioning; Veh, vehicle.

NAc core (NAcc), and NAc shell (NAcs) (13,15). Compared with vehicle, 7.5 mg/kg CBD significantly decreased Fos protein expression in the PL but not the IL, NAcc, or NAcs (Figure 3). Kruskal-Wallis tests confirmed that the density of Fos+ cells was significantly different across the experimentally naïve, control, vehicle-treated, and CBD-treated groups in the IL ($H_2 = 19.83, p < .001$), PL ($H_2 = 20.04, p < .001$), NAcc ($H_2 = 16.71, p < .001$), and NAcs ($H_2 = 15.43, p < .001$). Effect sizes for the IL, PL, NAcc, and NAcs were 0.63 (IL), 0.65 (PL), 0.53 (NAcc), and 0.50 (NAcs). Post hoc comparisons revealed a significant reduction in the number of Fos+ cells limited to the PL of CBD-treated rats compared with that of the vehicle group ($p < .05$) (Figure 3B). In both vehicle- and CBD-treated rats, higher Fos protein immunoreactivity was found across all 4 brain regions compared with naïve and control rats ($p < .05$). However, relative to no-test control rats, Fos protein expression was higher in the IL, NAcc, and NAcs, but not in the PL of CBD-treated rats (not significant) (Figure 3B).

In contrast to the behaviorally effective CBD dose (7.5 mg/kg), the behaviorally inert low (2.5 mg/kg, $n = 8$) and high (15 mg/kg, $n = 8$) doses did not reduce Fos+ cell density. However, relative to vehicle control rats, Fos+ cell density was significantly diminished ($p < .05$) in brain tissue of rats treated with 7.5mg/kg CBD (post hoc pairwise comparison following Kruskal-Wallis: $H_4 = 32.63, p < .001$) (Figure 4). The reduction in Fos+ cell density at the 7.5-mg/kg dose was statistically identical to that of the naïve and no-test control groups.

CBD Produces Decreases in Neuronal Activation of Both *Vglut+* and *Vgat+* Neurons

The PL contains both excitatory and inhibitory neuronal populations (25,26). To establish whether the observed reduction of PL neuronal activity by the behaviorally effective CBD dose (7.5 mg/kg) is selective for excitatory versus inhibitory neurons, in situ hybridization (RNAscope) was employed to quantify *Vglut*-mRNA-expressing, *Vgat*-mRNA-expressing, and *Chat*-mRNA-expressing neuronal subtypes (Figures 5 and 6). Consistent with the Fos protein data (Figures 3 and 4), quantitative analysis of *c-Fos* mRNA revealed a significant decrease in *c-Fos*-expressing cells within the PL of CBD-treated rats compared with vehicle control rats (Mann-Whitney; $p < .05$) (Figure 5C). The number of excitatory (glutamatergic) and inhibitory (GABAergic and glycinergic) neurons among the total DAPI cell population in the PL was similar (~40% *Vglut+* and ~5%–7% *Vgat+*) for both vehicle and CBD groups (Figure 5D). Analysis of activated neurons of distinct subtypes revealed a statistically significant decrease in activation by CBD among both the excitatory (*Vglut+*) and inhibitory (*Vgat+*) subpopulations (Mann-Whitney *U* tests; $p < .05$) (Figure 6D).

DISCUSSION

The results document that CBD significantly suppresses the expression of preference for a cocaine-paired environment (CPP) but according to a distinct U-shaped profile, with statistically reliable effects only at an intermediate dose.

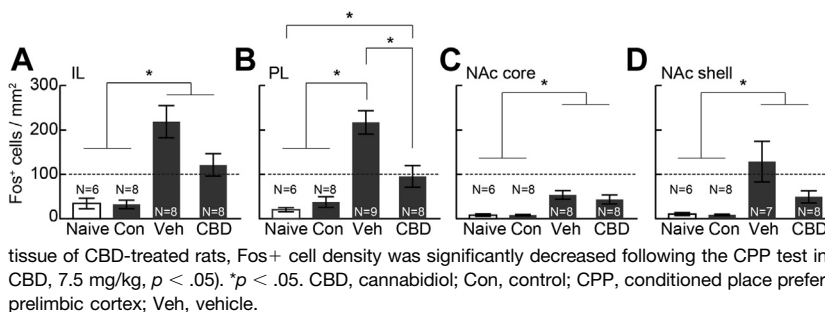


Figure 3. Effects of CBD on neuronal activation associated with expression of cocaine CPP. **(A–D)** Mean \pm SEM Fos+ cell density of naïve, no-test control, vehicle, and CBD (7.5 mg/kg) treated rats in **(A)** IL, **(B)** PL, **(C)** NAc core, and **(D)** NAc shell. Fos+ cell density was increased following CPP testing in all 4 brain regions (Veh vs. naïve, $p < .05$); however, exclusively in the PL, Fos+ cell density in the CBD group was statistically indistinguishable from that of the no-test control group ($p = .2$). In brain tissue of CBD-treated rats, Fos+ cell density was significantly decreased following the CPP test in the PL, but not in the IL, NAc core, or NAc shell (Veh vs. CBD, 7.5 mg/kg, $p < .05$). * $p < .05$. CBD, cannabidiol; Con, control; CPP, conditioned place preference; IL, infralimbic cortex; NAc, nucleus accumbens; PL, prelimbic cortex; Veh, vehicle.

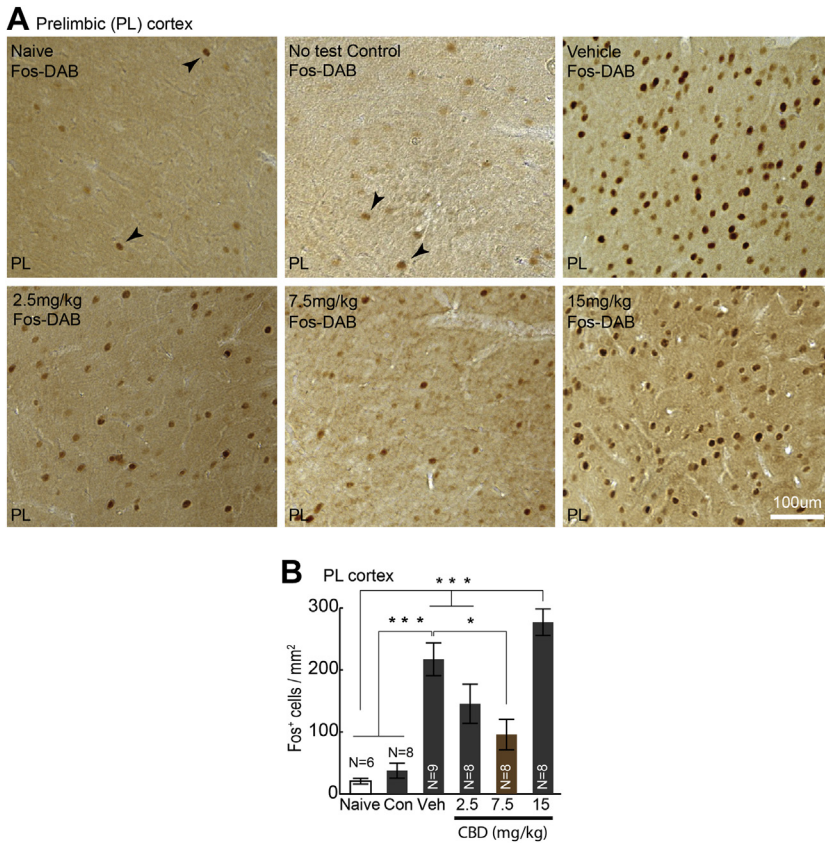


Figure 4. Neuronal activation in the PL. **(A)** Brightfield images of Fos+ cells visualized by DAB in the PL of naïve, no-test control, vehicle-treated, and CBD-treated rats with the low, intermediate, and high CBD dose. **(B)** Dose-dependent effects of CBD on Fos+ cell density following conditioned place preference testing. Mean \pm SEM numbers of Fos+ cells were reduced by CBD in a U-shaped manner with significant effects at the 7.5-mg/kg dose but not the 2.5-mg/kg or 15-mg/kg dose ($p < .05$ vs. vehicle; $p < .001$ vs. naïve/Con). * $p < .05$, *** $p < .001$. Con, control; CBD, cannabidiol; PL, prelimbic cortex.

CBD produced identical U-shaped dose-response profiles on neuronal mechanisms presumed to participate in mediating the drug's effects on CPP, with significant effects coinciding with the behaviorally active CBD dose. These neurobiological actions included suppression of CPP-associated neuronal activation in the PL (but not IL, NAcc, or NAcS), selective suppression of CPP-associated

activation of *Vglut+* and *Vgat+* neurons. These convergent dose-response profiles, in conjunction with the suppression of *Vglut-* and *Vgat-* neurons by the behaviorally most effective CBD dose implicate glutamatergic, GABAergic, and/or glycinergic populations in the PL as candidate neuroanatomical effector mechanisms for CBD's suppressant effects on CPP.

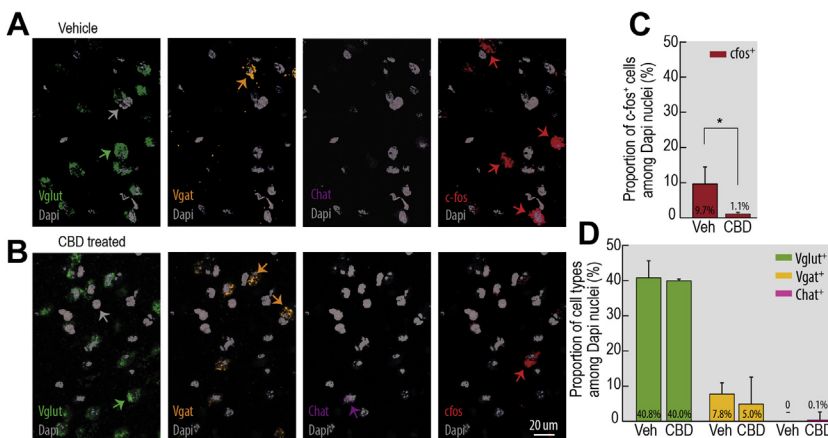


Figure 5. Effects of the behaviorally effective CBD dose (7.5 mg/kg) visualized by in situ hybridization RNAscope. **(A)** Confocal images of PL sections from a representative vehicle-treated rat showing *Vglut+* (green), *Vgat+* (orange), *Chat+* (magenta), and *c-Fos+* (red) mRNA-expressing cells. **(B)** Confocal images of PL sections from a representative CBD-treated rat showing an overall reduction of *c-Fos* mRNA in the PL compared with (top panel) the PL of the vehicle rat. All sections were stained with DAPI (gray pseudocolor). Note: Each punctate dot signal in panels **(A)** and **(B)** represents a single target RNA molecule. **(C)** Mean \pm SEM numbers of *c-Fos*+ cells among the total DAPI population. The number of activated cells following CPP testing was significantly reduced in the PL of CBD-treated rats compared with vehicle controls. ($p < .05$). **(D)** Mean \pm SEM numbers of *Vglut+*, *Vgat+*, or *Chat+* cell types presented as percent of the total DAPI population. * $p < .05$. CBD, cannabidiol; CPP, conditioned place preference; PL, prelimbic cortex; mRNA, messenger RNA; Veh, vehicle.

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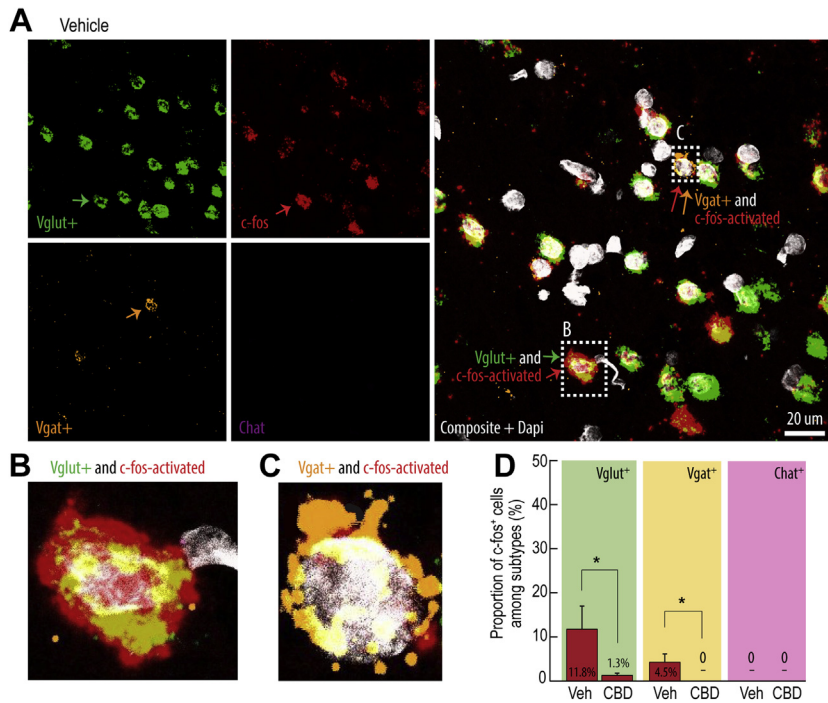


Figure 6. Effects of the behaviorally effective CBD dose (7.5 mg/kg) on activation of neuronal phenotypes in the PL as determined by RNAscope. **(A)** Confocal images of PL sections from a representative vehicle-treated rat showing *Vglut*+ (green), *c-Fos*+ (red), *Vgat*+ (orange), and *Chat*+ (magenta) mRNA-expressing cells, and a composite image visualizing these cell-type markers plus (larger panel) DAPI pseudocolored in white. **(B)** Inset from composite image in panel **(A)** but magnified to show colocalization of *Vglut* and *c-Fos* mRNA representing an active glutamatergic PL neuron. **(C)** An activated inhibitory PL neuron confirmed by visualizing colocalization of *Vgat* and *c-Fos* mRNA. Note: Each punctate dot signal in panels **(A)**, **(B)**, and **(C)** represents a single target RNA molecule. Multiple dots adjacent to one another give a fused appearance in microscope images. **(D)** Activated (*c-Fos*+) cells among the *Vglut*+, *Vgat*+, or *Chat*+ subpopulations. CBD significantly reduced the proportion of activated (*c-Fos*), glutamatergic (*Vglut*), and inhibitory (*Vgat*+) cells in the PL ($p < .05$ vs. Veh). * $p < .05$. CBD, cannabidiol; PL, prelimbic cortex; mRNA, messenger RNA; Veh, vehicle.

CBD Suppresses Cocaine CPP With a Distinct U-Shaped Dose-Response Profile

CBD reduced the time spent in the cocaine-paired compartment during the CPP test with a U-shaped dose-response profile characterized by significant suppression of CPP only at a single intermediate dose and disappearance of efficacy at the ascending limb of the U-shaped curve, but without increasing CPP at the highest dose (Figure 2). CBD did not significantly modify locomotor activity at any dose (Figure S2), such that the suppression of cocaine CPP cannot be explained by motoric effects of CBD. These findings unequivocally confirm U-shaped dose-response effects on cocaine-induced conditioned behavior, as tentatively implied by earlier findings in which an intermediate but not high CBD dose suppressed cocaine-induced CPP in mice following intraperitoneal administration (3) and attenuated conditioned fear responses following discrete intra-PL microinjection (10). It must be noted that dose-response profiles for CBD effects on addiction-related behavior in the literature include apparently linear profiles [for review, see (7)]. For example, intraperitoneal CBD reduced methamphetamine self-administration at 20, 40, and 80 mg/kg CBD with linear dose-response effects and attenuated methamphetamine-primed relapse-like behavior at the highest dose in male Sprague Dawley rats (27) [although intraperitoneally administered CBD failed to attenuate cocaine self-administration at either a 5 or 10 mg/kg dose in male Long Evans rats (28)]. Based on the present results and related evidence from the fear literature, U-shaped dose effects seem to be associated with conditioned behavioral responses such as CPP (in male mice) (3) and conditioned

fear (in male Wistar rats) (10) rather than primary drug reinforcement (i.e., self-administration) (3,27). The present findings illustrate that, when using a sufficiently wide dose range, distinct U-shaped dose-response effects emerge for CBD's attenuation of preference for the cocaine-paired environment, corroborated by U-shaped dose-response profiles of changes in associated activity-dependent neuronal activation. Although CPP represents a measure of Pavlovian conditioning, the U-shaped dose-response profile observed here may extend to operant conditioning models of drug seeking and relapse such as conditioned or context-induced reinstatement. Tentative support for this possibility comes from our earlier observations that maximal attenuation of context-induced reinstatement of cocaine and alcohol seeking by CBD occurred well before CBD levels in brain and plasma reached peak, and drug seeking did not decrease further despite rising CBD brain and plasma concentrations [(1) and unpublished observations (F. Weiss, Ph.D., et al., unpublished data, September 2019)], possibly suggestive of an underlying U-shaped function in CBD's effects on drug seeking in reinstatement models of relapse. Whether or not this is the case, the results obtained with the CPP procedure used here have direct translational relevance in their own right in light of evidence that the pairing of an environment with psychostimulant administration increases the subjective preference for the drug-paired environment in human subjects (29).

U-shaped dose-response profiles for CBD have been reported in animal and human studies of anxiety and psychiatric disorders (11,30–32), but they have not yet been an explicit focus of research on CBD's effects on behavior motivated by drugs of abuse. Studies of CBD effects on drug-induced CPP,

drug seeking, and drug taking have typically been limited to 1 or 2 doses (7). As well, without exception, these studies contain no information on brain or plasma concentrations associated with the behavioral effects of CBD, further complicating the evaluation of CBD's actions on addiction-relevant behavior in animal models of drug-motivated behavior and, thus, its SUD treatment potential. A U-shaped dose-response profile of CBD on drug-seeking behaviors would seem to require careful consideration for the choice of dosing regimens and identification of effective doses in SUD treatment approaches. Additionally, U-shaped dose-response effects may provide a basis for understanding the often discrepant findings on CBD effects on behaviors motivated by drugs of abuse in the literature [e.g., (7)].

CBD Reverses CPP-Associated Neuronal Activation of PL Neurons in a U-Shaped Manner

CBD significantly reduced CPP-associated neuronal activation, measured by Fos⁺ cell density, in the PL but not in the IL, NAcc, or NAcS (Figure 3). CBD's suppressant effects on neuronal activation in the PL followed the U-shaped profile of its behavioral effects: rats treated with the behaviorally effective CBD dose showed robust reduction in PL neuronal activity, whereas neuronal activation remained indistinguishable from vehicle control rats in rats treated with the behaviorally inert low and high doses (Figure 4B). Moreover, neuronal activation in the no-test control group was similar to that of naïve rats, indicating that neuronal activation was evoked by the cocaine-associated environment. This observation strongly suggests that the CBD-induced reduction of CPP-associated neuronal activation in the PL is functionally relevant for the observed behavioral effects of CBD (i.e., attenuation of cocaine-induced CPP). The suppression of PL neural activity observed here is consistent with findings showing that CBD concomitantly reduces conditioned fear and neuronal activation in the PL (10). In the latter study, Fos⁺ cell density was reduced in both the PL and IL; however, similar to the present findings, this reduction was significant only in the PL. Thus, whereas CBD tended to produce an overall decrease in PL, IL, and NAcS neuronal activation with similar effect sizes, statistically significant suppression was found only in the PL. Similar findings were reported by Lemos *et al.* (10), with both the PL and IL of CBD-treated rats showing diminished *c-Fos* activation; however, this CBD-induced decrease in activation was statistically significant only for the PL region (10). Therefore, given the present findings, as well as the observations by Lemos *et al.*, it appears that CBD-induced suppression of *c-Fos* activation is most prominent in the PL. Nonetheless, in view of the similar effect sizes across brain regions, a role of IL and NAcS neurons as additional behaviorally relevant targets of CBD actions warrants further scrutiny in the future.

CBD Suppresses Prelimbic Neuronal Activation of *Vglut+* and *Vgat+* Neurons

The PL contains heterogeneous neuronal populations (25,33). It was therefore essential to establish whether specific neuronal population(s) were recruited by CBD in association with its suppressant effects on cocaine CPP. This was accomplished by identifying the phenotypes of PL neurons

showing CBD-induced reduction in activation. RNAscope in situ hybridization in brain tissue of rats tested at the behaviorally effective CBD dose confirmed the initial Fos immunohistochemical findings with an overall decrease of *c-Fos* mRNA in the PL (Figure 5C). More importantly, these analyses established that the CBD-induced reduction in neuronal activation occurred in both *Vglut+* and *Vgat+* neurons (Figure 6D). Corroborating other reports, *Chat*-expressing subpopulations were extremely sparse (34–36). Only 2 *Chat+* neurons were encountered in the RNAscope analysis (Figure 5D), and neither of these *Chat+* neurons were *c-Fos* activated (Figure 6D). This finding provides evidence that CBD exerts a general inhibitory action on both *Vglut+* and *Vgat+* neuron subtypes in the PL. However, the mechanisms underlying the inhibition of individual cell types and how this inhibition is linked to CBD-induced reduction in cocaine CPP remain to be established. The PL has been widely implicated in cocaine relapse-like behavior and cocaine-induced CPP (37,38). The present findings provide an enhanced “resolution” of the PL role in cocaine-induced CPP by identifying the engagement of the *Vglut+* and *Vgat+* PL subpopulations in both the expression of cocaine-induced CPP and the suppressant effects of CBD on this behavior.

Conclusions

Transdermal CBD administration produced linear increases in CBD brain and plasma concentrations across doses but suppressed cocaine-induced CPP with a distinct U-shaped profile. A concomitant U-shaped dose effect of CBD on CPP-associated neuronal activation confirmed that behaviorally relevant pharmacological actions of CBD are reflected by corresponding dose-dependent effects on underlying brain function. Subsequent multiplex in situ hybridization identified suppression of glutamatergic as well as GABAergic (and/or glycinergic) signaling in the PL as a potential mechanism for the attenuation of cocaine-induced CPP by CBD. These findings inform future mechanistic efforts to establish the cellular and molecular modes of action by which CBD interferes with behavior motivated by cocaine and possibly other drugs of abuse. Also, the approaches taken here may guide future studies interrogating possible sex differences in the dose-response profile of CBD's behavioral and neuronal effects since the present findings are limited to male rats. Furthermore, to better understand CBD's actions on drug-motivated behavior, it will be important to establish whether the dose-response profile and effective dose range of CBD extends to behavior motivated by other substances of abuse. Overall, the findings continue to provide preclinical support for CBD's potential in the prevention of drug seeking and relapse. However, CBD's dose-response profile may have important implications for dosing regimens in clinical applications as well as the understanding of CBD effects (or lack thereof) on drug seeking in the literature.

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U-Shaped Cannabidiol Dose-Response Profile

FW conceived the research. FW and NS designed the research. HN and FW wrote the manuscript with contributions by GEW. NS coordinated and supervised all experiments. TMK performed the plasma and brain CBD concentration assays. GEW conducted the behavioral experiments assisted by AC, JW, and SZ. GDN performed RNAscope in situ hybridization. HN, GLDN, AC, SC, AHT, and NEE performed histology and brain image analyses. HN designed the semiautomated quantitative analysis. HN and FW analyzed the data.

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ARTICLE INFORMATION

From the Department of Neuroscience (HN, GEW, GLD, AC, TMK, JW, SZ, SC, AHT, NEE, NS, FW), The Scripps Research Institute, La Jolla, California.

HN and GEW contributed equally to this work as joint first authors.

NS and FW contributed equally to this work as joint senior authors.

TMK is currently affiliated with the College of Pharmacy, The University of Texas at Austin, Austin, Texas.

Address correspondence to Friedbert Weiss, Ph.D., at bweiss@scripps.edu, or Nobuyoshi Suto, Ph.D., at nsuto@scripps.edu.

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