Tolerance to the diuretic effects of cannabinoids and cross-tolerance to a kappa-opioid agonist in THC-treated mice

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Non-standard abbreviations

AM4054: 9β-(Hydroxymethyl)-3-(1-adamantyl)-hexahydrocannabinol
THC: Δ9-Tetrahydrocannabinol
U50,488: trans-(--)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide
MED: Minimum effective dose
%MPE: Percent of maximum possible effect
ABSTRACT

Daily treatment with cannabinoids results in tolerance to many, but not all, of their behavioral and physiological effects. The present studies investigated the effects of 7-day exposure to 10 mg/kg/day Δ⁹-tetrahydrocannabinol (THC) on the diuretic and antinociceptive effects of THC and the synthetic cannabinoid AM4054. Comparison studies determined diuretic responses to the κ-opioid agonist U50,488 and furosemide. Following determination of control dose-response functions, mice received 10 mg/kg/day THC for 7 days, and dose-response functions were redetermined 24h, 7 days, or 14 days later. THC and AM4054 had biphasic diuretic effects under control conditions with maximum effects of 30 and 35 ml/kg urine, respectively. In contrast, antinociceptive effects of both drugs increased monotonically with dose to >90% MPE. Treatment with THC produced 9- and 3-fold rightward shifts of the diuresis and antinociception dose-response curves for THC and, respectively, 7- and 3-fold rightward shifts in the AM4054 dose-response functions. U50,488 and furosemide increased urine output to >35 ml/kg under control conditions. The effects of U50,488 were attenuated following 7-day treatment with THC, whereas the effects of furosemide were unaltered. Diuretic effects of THC and AM4054 recovered to near-baseline levels within 14 days after stopping daily THC injections, while tolerance to the antinociceptive effects persisted longer than 14 days. The tolerance induced by 7-day treatment with THC was accompanied by a 55% decrease in the B_{max} value for cannabinoid CB1 receptors. These data indicate that repeated exposure to THC produces similar rightward shifts in the ascending and descending limbs of cannabinoid diuresis dose-effect curves, and to antinociceptive effects, while resulting in a flattening of the U50,488 diuresis dose-effect function.
INTRODUCTION

Marijuana and other cannabis products historically have been illegal in most countries, although their regulated medicinal or recreational use is becoming more prevalent (UNODC, 2014). The use of cannabis products (hereafter referred to by their principal psychoactive constituent, \(\Delta^9\)-tetrahydrocannabinol, or THC) often is characterized by repeated or long-term intake, whether as recreational drugs or for their reported medicinal effects in managing chronic conditions including pain, anxiety, nausea, and anorexia (Pacher et al., 2006). Consequently, a full understanding of THC’s pharmacology necessitates the evaluation of how its effects are altered by repeated or chronic administration.

Repeated exposure to THC or other cannabinoid agonists in vivo has been shown to produce both down regulation and desensitization of CB1 cannabinoid receptors, leading to decreases in downstream signaling processes (Breivogel et al., 1999; Sim-Selley and Martin, 2002; Sim-Selley et al., 2006). These changes in CB1 receptor signaling are consistent with observations in earlier behavioral studies that demonstrated tolerance to the effects of THC or other CB1 receptor agonists in the four ‘tetrad’ effects originally used to characterize cannabinoid drugs (Fan et al., 1994; Martin et al., 1991). However, the development of tolerance is not always uniform, nor necessarily evident across all behavioral endpoints. For example, mice treated daily with the CB1 agonists JWH-018 or JWH-073 demonstrated a complete loss of hypothermic responses following the second daily injection, whereas locomotor suppression induced by these drugs continued throughout the period of daily injections (Tai et al., 2015). Moreover, the magnitude of cannabinoid tolerance and cross-tolerance can vary with either the drug given chronically or the drug tested acutely (Fan et al., 1994). In one example of selective cross-tolerance, 32 mg/kg/day THC eliminated the
hypothemic effects of both THC and CP 55,940, and produced a rightward shift of the THC dose-response curve for rate-decreasing effects without altering the response-suppressing effects of CP 55,940 on operant behavior (Singh et al., 2011). Conventional receptor theory has been invoked to attribute such differences in the expression of cross-tolerance among drugs to documented differences in their efficacy. From this perspective, partial agonists that require greater receptor occupancy to produce their effects, like THC, are more likely to induce and show tolerance than are putative full agonists, such as CP55, 940 and JWH-018, which should have greater receptor reserves (Hruba et al., 2012). Thus, although considerable tolerance may develop to the behavioral effects of cannabinoids, its magnitude and time course may differ according to the drugs and endpoints under study.

We recently reported that cannabinoid-induced diuresis is a CB1-receptor mediated effect in rodents and, additionally, that these diuretic actions in mice are biphasic (Chopda et al., 2013; Paronis et al., 2013). Diuretic effects of cannabinoids were first reported anecdotally, and subsequently confirmed, in early studies of cannabis in humans (Ames, 1958; Parker and Wrigley, 1947). Yet, diuresis in human subjects is not frequently mentioned in existing literature, suggesting that despite the widespread use of cannabis products, most people either do not notice or do not complain about THC-related diuresis. Given that marijuana exposure in humans often continues over years or even decades (e.g., (Budney et al., 2003; Copersino et al., 2006), it may be that tolerance develops to the diuretic effects of marijuana and other cannabinoids. With this in mind, the present studies were conducted to evaluate changes in the diuretic effects of two cannabinoids, the CB1 partial agonist THC and the CB1 putative full agonist AM4054, consequent to a 7 day dosing regimen with 10 mg/kg THC. In particular, given the curvilinear nature of the cannabinoid diuresis function in mice, we were interested in
determining whether both the ascending and descending limbs of the function are susceptible to tolerance. For comparison, the diuretic effects of the \( \kappa \)-opioid agonist U50,488 and the loop diuretic furosemide also were determined in mice exposed to the same course of daily THC administration. Additional experiments were conducted to provide information on the effects of 10 mg/kg/day THC administration on a more conventional cannabinoid-induced effect, antinociception, as well as on cannabinoid CB1 receptor binding in mouse cerebellum.
METHODS

Subjects. Male CD-1 mice (Charles River Laboratories, Wilmington MA) weighing 20-25 g at the start of the study, were housed 4/cage in a climate controlled vivarium with food and water available ad libitum. Mice were acclimatized to the study procedures twice prior to testing; each mouse was used in only one type of assay. All experiments were performed during the light portion of the light/dark cycle. All studies were approved by the Northeastern University Animal Care and Use Committee, in accordance with guidelines established by the National Research Council.

Diuresis Procedures. Mice were injected s.c. with vehicle or drug solutions using a constant volume of 1ml/100g. Immediately after injection, and without abdominal manipulation, they were placed on an elevated grid floor and isolated under a plastic cup (10cm x 5cm; d x h). Weigh boats placed underneath each mouse collected voided urine, and these were weighed every 2 h over a total period of 6 h. All mice were tested with saline prior to any drug or vehicle exposure. After this initial test, 120 mice were divided into 4 groups used to examine effects of THC or its vehicle, AM4054, U50488, or furosemide. All mice were tested once before and 1 or 2 times after daily THC exposure with the same drug, with the exception that mice in the furosemide group were tested with one of the other drugs at 7 or 14 days after THC exposure. Dose selection for individual mice varied and the effects of each dose were determined in 6 to 10 animals, except where noted.

Antinociception Procedures. Antinociception was determined using a warm water tail-withdrawal assay. A water bath maintained water temperature at 52.0 ± 0.5°C. Each mouse was gently hand held and the distal 2-3 cm of the tail was immersed in the water; latency to tail-withdrawal was measured using a stopwatch, and a cut-off time of 8s was established to avoid
tissue damage. Baseline latencies were determined twice on each test day with a 10 min interval between determinations. Mice were divided into three groups of 8 animals. One group of animals was used to evaluate the time course of the antinociceptive effects of AM4054; these mice were tested once per week with all doses of AM4054 given in a randomized order. In the remaining two groups of animals, complete dose-response curves for either THC or AM4054 were generated in each mouse using cumulative dosing procedures similar to those described previously (Paronis and Woods, 1997). Briefly, 60 min after an injection, tail-withdrawal latencies were determined and mice were then injected with the next dose, such that the total cumulative dose was increased by 0.25 or 0.5 log units. This procedure was repeated until the tail-withdrawal latency reached the cut-off or no longer increased with subsequent increases in drug dose. Dose-response curves were determined in all animals prior to THC treatment and at 24 h, 7 days, and 14 days after 7 daily injection of 10 mg/kg THC.

**Binding Procedures.** Two groups of mice (n = 6) were injected daily with vehicle or 10 mg/kg THC for 7 days. At 24 h after the last injection, mice were sacrificed using cervical dislocation; whole brain was then collected and frozen at -80°C until further analysis. On the day of the binding assay and using methods previously described (Gatley et al., 1998; Gifford et al., 1999), the cerebellum from each brain was isolated, weighed, and separately homogenized in TME (100 mM Tris, 5 mM MgCl₂, 1 mM EDTA) buffer containing 3% BSA to obtain a 10 mg/ml homogenate. 3 ml homogenate was transferred to another tube to which 1.5 μCi [¹²⁵I]AM281 was added. Binding assays were completed by incubating 100 μl radiolabeled homogenate with 100 μl of TME buffer or 1 pM - 10 μM AM251 and 800 μl homogenizing buffer for 90 min at room temperature on a shaker, followed by centrifugation (14000 rpm) for 12 min at 4°C. The supernatant was removed and pellets were blotted dry. Radioactivity was
measured with a gamma counter; specific activity and total radioactivity were determined using 100 μl of homogenate plus \[^{125}\text{I}\]AM281. All samples were run in duplicate.

**Drugs and Dosing Procedures.** \(\Delta^9\)-THC was obtained from the National Institute on Drug Abuse [(NIDA), Rockville, MD]; U50,488 [trans-(+/-)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]-cyclohexyl)-benzeneacetamide methane sulfate] and furosemide were purchased from Sigma-Aldrich (St. Louis, MO). AM4054 [9\(\beta\)-(hydroxymethyl)-3-(1-adamantyl)-hexahydrocannabinol] was synthesized at the Center for Drug Discovery, Northeastern University. U50,488 was dissolved in saline. Furosemide was dissolved in 1% 1N NaOH and sterile water. THC and AM4054 were initially dissolved in ethanol to which an equal volume of emulphor-620 (Rhodia, Cranbury, NJ) was added, and then further diluted with saline to achieve a 1:1:18 mixture; this 1:1:18 solution, without drug, is referred to as vehicle. Drug doses are expressed in terms of the weight of free base and injections were delivered s.c. in volumes of 1 ml/100g body weight. All drug injections were given between 8-10AM.

**Data Analysis.** Urine volumes were normalized to volume (ml) per kg bodyweight. Data obtained after saline injection in individual animals were grouped according to the drug tested and averaged to obtain group means, SEM, and 99% CI. Minimum effective doses (MED) were defined as those found 0.5 ml/kg outside the 99%CI of the mean saline values, calculated using interpolation of two data points found above and within or below the 99% CI. Tail withdrawal latencies are expressed as a percentage of maximum possible effect (%MPE ± SEM), calculated using the formula: \(%\text{MPE} = [(\text{test latency} - \text{baseline latency})/ (8 - \text{baseline latency})] \times 100.\) Antinociception ED\(_{50}\) values (with 95%CI) were calculated from grouped data using linear regression of data points \(\geq 10\%\) MPE. Potency ratios were calculated by dividing the baseline dose value from the equieffective dose value following daily THC exposure.
Binding data were normalized to the protein content of the brain homogenate. Specific binding was determined by subtracting nonspecific binding from total binding, and $B_{\text{max}}$ values (± SEM) were determined using Scatchard analysis (GraphPad Prism v.5.02, GraphPad Software, La Jolla, CA). All drug data were plotted and analyzed using log transformed values of doses. Data were analyzed using paired t-tests, one-way, or two-way ANOVA followed by Dunnett’s or Bonferroni’s multiple comparison tests; significance for all tests was set at $p < 0.05$. 
RESULTS

Cannabinoid Diuresis. Saline injection resulted in an average of 16.3±0.9 ml/kg voided urine over 6 h for all mice, and no significant differences were found among different groups of animals [F(3,16) = 1.754, ns]. Injection of the cannabinoid vehicle had effects similar to saline, and 6 daily injections of vehicle did not alter diuretic responses measured on alternate days of treatment [F(2,14) = 0.566, n.s] (see Table 1). Under control conditions, THC and AM4054 had curvilinear dose-response curves, dose-dependently increasing, then decreasing, urine output. For THC, doses of 1.0-10.0 mg/kg formed an ascending limb of the curve, and doses of 10-100 mg/kg formed a declining limb of the function. Similarly, dose-related diuresis occurred following 0.01-0.3 mg/kg AM4054, and 1.0 mg/kg resulted in urine output below saline levels (Figure 1).

The diuretic effects of daily injection of 10 mg/kg THC were measured on alternate days in a group of 8 mice. This dose of THC initially produced a mean urine output of 32.6 ml/kg, and these effects decreased gradually with daily THC administration (see Table 1). After the 7th daily injection of THC only 14.3 ml/kg urine was voided, similar to the effects of saline injection and significantly different from the effects observed after the first injection of 10 mg/kg THC [F(3,21) = 5.618, p<0.01]. In comparison, in mice that had received repeated vehicle injections, the response to a single injection of 10 mg/kg THC on the 7th day was similar to the effects on Day 1, and significantly different from those on Day 7, of the THC-treated mice [F(2,21) = 6.047, p<0.01] (Table 1).

Shown in Figure 1A, a THC dose-response curve generated at 24 h after the last (7th) daily injection of THC revealed a rightward shift in the ascending limb of the function, reflected in a 10-fold increase in the peak dose of THC, as well as in the lowest effective dose
(Table 2). The descending limb of the THC dose response curve also appeared to shift rightward, however, the effects of THC doses higher than 100 mg/kg could not be determined as the vehicle required for high THC concentrations had measurable antidiuretic effects (unpublished observations). A two-way ANOVA on the effects of 10-100 mg/kg THC revealed a significant interaction between dose and time \( [F(4,71)=5.475, p<0.01] \), and post-hoc analysis indicated differences in urine volume of THC-treated mice following 10 and 100 mg/kg THC, compared to control conditions. There were no differences in the diuretic responses to 30 mg/kg THC, though notably this dose was found on the descending limb of the function under control conditions, and on the ascending limb of the function after daily THC treatment. At 14 days after ending daily THC injections the ascending limb of the THC dose-response curve remained to the right of the control dose-response curve (Figure 1A). Although maximum urine volumes obtained at 24 h or 14 days after daily THC treatment were marginally higher or lower, respectively, then control values, these difference were not significant \( [F(2,25)=2.521, \text{ns}] \).

Both limbs of the AM4054 dose response curve were shifted to the right following daily THC treatment (fig. 1B). The lowest effective dose calculated for AM4054-induced diuresis was 0.05 mg/kg under control conditions, and the lowest dose that produced less diuresis than saline was 0.54 mg/kg. At 24 h after the last daily injection of THC, both MED values for AM4054 were increased by 5- to 7-fold (Tables 2 and 3). A two-way ANOVA on the effects of 0.1-1.0 mg/kg AM4054 revealed a significant interaction between dose and time \( [F(4,44)=10.19, p<0.01] \), and post-hoc analysis indicated differences in urine volume of THC-treated mice following 1.0 mg/kg AM4054, compared to control conditions. There was partial recovery in the position of both limbs of the AM4054 diuresis dose-response curves 7 days after the last daily THC injection, as the MED values were 2-fold higher than those obtained under control
conditions. There was a full recovery to control effects of AM4054 by 14 days after ending daily THC injections (fig. 1B). The maximum urine outputs produced by AM4054 administration were not statistically different from control values [$F_{(3,19)}=1.639$, ns] (see Table 2).

**Non-cannabinoid Diuresis.** The ability of THC treatment to alter other diuretic responses were evaluated in mice tested with saline, furosemide, or U50,488. Daily THC exposure did not alter diuretic responses to saline or to furosemide (Table 4). In contrast, the diuretic effects of U50,488 were diminished in THC-treated mice at 24 h and 7 days after ending THC injections (see Table 2 and Figure 2). The MED values for U50,488 were higher in THC-treated mice, relative to control values, however, changes in the U50,488 dose-response function were different from the effects seen in the THC and AM4054 dose-response curves. In contrast to effects obtained with cannabinoid agonists, the peak diuretic effects of U50,488 were decreased as a function of THC-treatment, yet the dose producing peak effects, 30 mg/kg, was unchanged [$F_{(3,44)}=5.1$, $p<0.01$]. Thus, daily injection of 10 mg/kg THC resulted in a flattening of the U50,488 dose-response curve that was evident for more than 7 days after the last injection of THC (Figure 2). As noted for both AM4054 and THC, by 14 days after ending the daily THC injections there was substantial recovery of the diuretic effects of U50,488.

**Antinociception.** Initial studies evaluated the time course of the antinociceptive effects of AM4054, to determine a suitable inter-injection interval for subsequent studies employing cumulative dosing with this drug. Averaged baseline tail-withdrawal latencies did not vary significantly across conditions [$F_{(3,28)}=1.782$, ns] and ranged from 1.8±0.2 to 2.2±0.1 sec. All doses of AM4054 tested reached near peak antinociceptive effects within 1 h of injection, and
these effects were sustained for at least 4 h (Figure 3); based on these results, a 1 h interjection interval was used for all cumulative dosing studies.

Averaged baseline tail-withdrawal latencies for mice tested with THC and AM4054 were 1.9±0.1 and 2.5±0.1 sec, respectively. Both cannabinoid drugs produced dose-related increases in antinociception under control conditions, with ED\(_{50}\) values (and 95%CI) of 13.2 (7.6, 22.9) and 0.3 (0.2, 0.5) mg/kg, respectively. The initial antinociceptive effects of 10 mg/kg THC were moderate, resulting in 38.7±10.1 %MPE in individual animals. These moderate effects were eliminated during daily treatment with 10 mg/kg THC; after the 3rd daily THC injection, the average antinociceptive effect was 14±4% MPE. The daily injection of 10 mg/kg THC produced a rightward shift of the full THC dose-response curve, yielding an ED\(_{50}\) value of 94.2 mg/kg (Fig. 4). There was little recovery towards baseline up to 14 days after ending the daily THC injections, and the potency ratio compared to control conditions was greater than 4-fold (Table 2).

Daily THC injections also produced a smaller, but significant, rightward shift of the dose-response curve for the antinociceptive effects of AM4054, and the mean ED\(_{50}\) value increased approximately 3-fold to 1.0 (0.7, 1.4) mg/kg. As observed with THC, there was no recovery of the AM4054 dose-response curve to its basal position, and the potency ratio was still 3-fold at 14 days after ending the daily THC injections.

**Binding.** CB1 receptor levels in cerebellum were compared in mice that received 7-day treatment with either vehicle or 10 mg/kg THC and were then sacrificed 24 h after the last injection (Figure 5). Mouse cerebellum has previously been shown to have a high density of CB1 receptors sites (Herkenham et al., 1991) that are sensitive to downregulation (McKinney et al., 2008). The \(B_{\text{max}}\) for CB1 receptors in vehicle-mice was 170 ± 30 pmol/mg. The \(B_{\text{max}}\) value
in mice that received daily THC was 75 ± 9 pmol/mg, significantly lower than $B_{\text{max}}$ values obtained from vehicle treated mice [$F_{(2,6)}=29.77$, $p<0.01$.].
DISCUSSION

The results of these experiments demonstrate that the diuretic effects of cannabinoid agonists are subject to tolerance. Moreover, for THC and AM4054 roughly equivalent tolerance developed to both diuretic and antinociceptive effects, providing further evidence that similar receptor mechanisms may underlie these two different cannabinoid effects (Chopda et al., 2013). These findings notwithstanding, there was a slight difference in the magnitude of tolerance to the two cannabinoid agonists and, additionally, the dose-response curve of AM4054 showed faster recovery than did the dose-response curve of THC. These observations are in keeping with the designation of THC as a partial CB1 agonist and AM4054 as a putative full agonist (Desai et al., 2013; Paronis et al., 2012). An unexpected finding of the present study was that chronic administration of 10 mg/kg/day THC also resulted in attenuation of the diuretic effects of the κ-opioid agonist U50,488. The effect of chronic administration of THC on the diuretic response to U50,488 suggests that prolonged exposure to cannabinoid agonists has effects that extend beyond the cannabinoid system and, more specifically, to the κ-opioid system.

Unlike other CB1 receptor-mediated effects, the dose-response curve for cannabinoid diuresis in mice consistently displays a biphasic function. An earlier study revealed that the increasing and decreasing effects of AM4054 on diuresis are both antagonized by rimonabant, although slightly greater antagonism was evident in the ascending limb of the function (Chopda et al., 2013). In contrast, the descending limb of the function was more sensitive to antagonism by AM6545, a CB1 antagonist with poor CNS penetration, suggesting involvement of peripheral mechanisms in the decreases in urine output (Chopda et al., 2013; Tam et al., 2010). Thus, it was of particular interest to determine whether both limbs of the diuresis dose-effect
curves for cannabinoids are similarly subject to tolerance. In THC-treated mice, the entire AM4054 dose-response curve showed a rightward displacement, and changes in the position of the THC dose response curve appeared qualitatively similar to those seen with AM4054. Moreover, the magnitude of the rightward shifts of the THC and AM4054 dose-response curves for diuresis approximate those observed for the antinociceptive effects of both drugs. These data, coupled with the 50% decrease in binding sites, are consistent with results of previous findings on cannabinoid tolerance in mice and extend them to include diuretic effects (Fan et al., 1994; McKinney et al., 2008; Nguyen et al., 2012; Singh et al., 2011). Of note, earlier studies typically used doses of THC ranging from 20 to 60 mg/kg/day to investigate the development of tolerance, with dosing regimens lasting from 2 to 13 days (Fan et al., 1994; Bass and Martin, 2000; McKinney et al., 2008). The present studies demonstrate that a one week exposure to a lower daily dosage of THC suffices to reveal substantial tolerance to cannabinoid effects, comparable to that reported using higher daily dosages.

The baseline positions of the ascending and descending portions of the AM4054 dose-response curve were recovered by 14 days after ending daily THC treatment, whereas the ascending limb of the THC dose response function remained to the right of the control position, as did the curves for antinociceptive effects of both agonists. Slow or incomplete recovery of full cannabinoid effects in THC-treated mice has been reported elsewhere, and recovery time varies with the effect measured. For example, Bass and Martin (2000) found full recovery of the locomotor activity-suppressant effects of THC within 7 days following cessation of 20 mg/kg/day THC, whereas the dose-response function for antinociceptive effects was recovered fully only after 12 days. Similarly, McMahon and colleagues reported an incomplete recovery for hypothermic and operant response rate-decreasing effects of CP55,940 and THC more than
10 days after stopping daily treatment with 32 mg/kg THC (Singh et al., 2011). In another study, a single intracerebroventricular administration of WIN 55,212 produced tolerance to the antinociceptive effects of the drug that lasted more than 14 days (Garzón et al., 2008). The variable, yet overall slow, return of dose-response curves to their baseline position following repeated exposure to THC may depend on the prolonged, regionally-dependent, recovery rate of CB1 receptor levels or, alternatively, to altered coupling to second messenger systems (Garzón et al., 2008; Sim-Selley et al., 2006).

Maximum urine volumes were marginally higher in mice tested with either THC or AM4054 at 24 h after ending daily THC treatment than under control conditions. This stands in marked contrast to the nearly complete attenuation of U50,488-induced diuresis measured at the same time point. This was the most unexpected result of our studies, both because the tolerance to U50,488 represents a cross-tolerance across different drug classes, and also because the expression of the effect – a downward- rather than rightward-shift in the dose-response curve - was different and in opposition to the changes seen in the effects of cannabinoids. THC and U50,488 both result in a free-water diuresis, suggesting that perhaps cannabinoids, like κ-opioid agonists, produce their diuretic effects centrally by suppressing vasopressin release (Chopda et al., 2013; Leander et al., 1987; Leander et al., 1985; Sofia et al., 1977). This raises the possibility that repeated THC exposure may alter circulating levels of vasopressin, however, in contrast to decreased effects of U50,488 and cannabinoid agonists, diuretic responses to furosemide or 10 ml/kg saline were unchanged in THC-treated mice. Together, these results indicate that the cross-tolerance induced by daily THC exposure does not extend to peripherally-acting loop diuretics and that fluid-handling, per se, was not affected by the daily THC injections. This suggests there is some specificity to the effects induced by daily THC,
although the mechanisms through which THC exposure reduces U50,488-induced diuresis are not clear.

Interactions between cannabinoids and κ-opioid opioids have been explored previously, based on their common signaling transduction mechanisms or their often similar behavioral effects (Hampson et al., 2000; Maguire and France, 2016; Walentiny et al., 2010). For example, the κ-opioid antagonist, nor-binaltorphimine, will block the antinociceptive effects of THC whereas co-administration of cannabinoid and κ-opioid agonists have additive antinociceptive effects (Maguire and France, 2016; Smith et al., 1994). Early reports of a bidirectional antinociceptive cross-tolerance between THC and κ-opioid agonists led to subsequent studies demonstrating the involvement of the endogenous κ-opioid, dynorphin, in the antinociceptive effects of THC (Pugh et al., 1996; Smith et al., 1994). Dynorphin and κ-opioid receptor activation also have been implicated in reducing the reinforcing effects of cannabinoid agonists in mice although blockade or removal of κ-opioid receptors does not alter other “tetrad” effects of THC (Ghozland et al., 2002; Mendizábal et al., 2006). It is conceivable that dynorphin also mediates the diuretic effects of cannabinoids, although preliminary studies from our laboratory found no effects of pretreatment with the opioid antagonist, naltrexone, on THC or AM4054-induced diuresis.

Other investigators have pursued more direct interactions between cannabinoids and κ-opioid receptors, demonstrating that CB1 and κ-opioid receptors co-localize, and potentially dimerize, in cultured cells, and also that the CB1 antagonists, rimonabant, is able to antagonize the effects of the κ-opioid agonist U69,593 on [35S]GTPγS binding in KOR-transfected HEK293 cells (Rios et al., 2006; Walentiny et al., 2010). Further, mice treated for 10 days with the cannabinoid antagonist AM281 showed increases in the expression of both prodynorphin
and κ-opioid receptor genes (Rios et al., 2006; Saez-Cassanelli et al., 2007). These latter studies open the possibility that repeated exposure to a cannabinoid agonist, such as THC, may induce a down-regulation within the κ-opioid system, resulting in decreased responsiveness to κ-opioid agonists. Alternatively, administration of THC may indirectly decrease κ-opioid receptor availability. Stimulated vasopressin release has been associated with a near doubling of κ-receptors in the plasma membrane of hypothalamic neurosecretory cells (Shuster et al., 1999). If cannabinoid agonists produce diuresis though inhibition of vasopressin release, this may be associated with decreased trafficking of κ-receptors and the resultant downward-shift in the U50,488 dose-response curve seen in the present studies. Although intriguing, this suggestion must remain speculative without further investigation, and notwithstanding similarities in the acute effects of these drugs, it remains difficult to explain the loss of U50,488 mediated diuresis in THC-treated mice, especially in comparison to the overall rightward shifts of the cannabinoid dose-response curves.

In summary, the present results confirm and extend earlier observations of cannabinoid tolerance by demonstrating that a relatively low dose of 10 mg/kg/day THC is adequate to produce antinociceptive tolerance that endures for at least 14 days. Using the same dosing regimen, we further show that roughly equivalent tolerance is produced to the diuretic effects of cannabinoids. Moreover, despite the curvilinear nature of the diuresis dose-effect curves, rightward shifts were obtained in both the ascending and descending limbs of the functions, suggesting the cannabinoid tolerance is receptor-mediated and surmountable. However, the daily exposure to THC also decreased the effects of a κ-opioid agonist, demonstrating that THC-induced tolerance extends beyond the cannabinoid system, specifically to the κ-opioid receptor system. The attenuation of U50,488-mediated diuresis was different in appearance, as
the dose response curve was shifted downward, not rightward like the cannabinoid dose response curves, yet the duration of the tolerance was similar to that expressed by THC and AM4054. Cannabinoid CB1 receptors are frequently cited as being among the most abundant G-protein coupled receptors, widely distributed within the central nervous system; thus, it is not unreasonable to anticipate that acutely administered cannabinoid agonists interact with ligands of other receptor systems (Gifford et al., 1999; Herkenham et al., 1991; Sim-Selley et al., 2006). Our findings suggest that, in addition to acute effects, repeated exposure to cannabinoid agonists also may profoundly alter the effects of drugs that produce their effects through other neurotransmitter systems.
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AUTHORSHIP CONTRIBUTION

Participated in research design: Chopda, Gatley, Paronis

Conducted experiments: Chopda, Parge

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Wrote or contributed to the writing of the manuscript: Chopda, Paronis
REFERENCES


Sim-Selley LJ and Martin BR (2002) Effect of chronic administration of R-(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN55,212-2) or delta(9)-tetrahydrocannabinol on cannabinoid receptor adaptation in mice. *J Pharm Exp Ther* 303:36-44.


FOOTNOTES

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Portions of this work were presented previously: a) in G.R.C.’s doctoral dissertation defense as follows: Chopda GR (2013) *Cannabinoid-mediated diuresis in mice*. Doctoral dissertation, Northeastern University, Boston, MA; b) as Sonti S, Chopda GR, Paronis, CA, Gatley, SJ. (2013). The effects of acute and chronic administration of delta-9-tetrahydrocannabinol (Δ⁹-THC) on receptor downregulation in mice. The 23rd Annual Symposium of the International Cannabinoid Research Society, Vancouver, Canada.

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FIGURE LEGENDS

Figure 1. Cannabinoid diuresis over 6 hr. Panel A: Effects of THC before (closed symbols) and 24 hr, or 14 day after 7-day THC administration; Panel B: Effects of AM4054 before (closed symbols) and 24 hr, 7 day, or 14 day after 7-day THC administration. Each point represents the mean of 6-10 mice (except n=3 following 10 mg/kg AM4054) and vertical bars indicate ±SEM. The shaded area in between dotted lines marks the 99%CI for urine volume following saline injection. Ordinates: urine output over 6 h in ml/kg body weight; Abscissae: drug dose in mg/kg.

Figure 2. Diuretic effects of U50,488 before (closed symbols) and 24 hr, 7 day, or 14 day after 7-day THC administration (n=6-10); other details as in figure 1.

Figure 3. Timecourse of the antinociceptive effects of AM4054. Ordinate: antinociceptive response, expressed as a percentage of maximum possible effect (%MPE). Abscissae: Time (in minutes) since injection of AM4054.

Figure 4. Antinociceptive effects of AM4054 and THC. Panel A: Effects of THC before and after 7-day THC administration (n=8); Panel B: Effects of AM4054 before and after 7-day THC administration (n=8). Ordinates: antinociceptive response, expressed as a percentage of maximum possible effect (%MPE). Abscissae: cumulative drug dose in mg/kg body weight.
Figure 5. Linear Scatchard transformations of the binding of $[^{125}\text{I}]$AM281 to cerebellum in mice sacrificed after 7-day vehicle administration (closed circles) or in mice sacrificed 24 h after 7-day THC administration (open squares).
Table 1. Urine output (in ml/kg, mean ± SEM) of mice injected daily with 10 mg/kg THC for seven days (THC Group, n=8) or mice injected daily with vehicle for 6 days and, on day 7, with 10 mg/kg THC (Vehicle Group, n=8).

<table>
<thead>
<tr>
<th></th>
<th>THC Group</th>
<th>Vehicle Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>32.6 ± 4.0</td>
<td>15.7 ± 2.9</td>
</tr>
<tr>
<td>Day 3</td>
<td>25.7 ± 4.8</td>
<td>12.5 ± 3.1</td>
</tr>
<tr>
<td>Day 5</td>
<td>18.1 ± 3.1*</td>
<td>14.6 ± 3.2</td>
</tr>
<tr>
<td>Day 7</td>
<td>14.3 ± 2.8**</td>
<td>31.1 ± 5.2##</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 compared to THC-Group, Day 1.

## p<0.01 compared to THC-Group, Day 5.
Table 2. Maximum urine outputs (mean ± SEM) and minimum effective doses (MED)\textsuperscript{a} calculated for ascending and descending limbs of diuresis dose response curves.

<table>
<thead>
<tr>
<th></th>
<th>Maximum output (ml/kg)</th>
<th>MED (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ascending limb</td>
</tr>
<tr>
<td>THC control</td>
<td>30.5 ± 3.6</td>
<td>2.2</td>
</tr>
<tr>
<td>24 h post-THC</td>
<td>36.3 ± 3.2</td>
<td>19.9</td>
</tr>
<tr>
<td>14 day post-THC</td>
<td>23.8 ± 3.8</td>
<td>8.0</td>
</tr>
<tr>
<td>AM4054 control</td>
<td>35.0 ± 5.7</td>
<td>0.05</td>
</tr>
<tr>
<td>24 h post-THC</td>
<td>42.0 ± 2.7</td>
<td>0.33</td>
</tr>
<tr>
<td>7 day post-THC</td>
<td>32.7 ± 2.9</td>
<td>0.11</td>
</tr>
<tr>
<td>14 day post-THC</td>
<td>29.2 ± 3.6</td>
<td>0.05</td>
</tr>
<tr>
<td>U50,488 control</td>
<td>37.0 ± 2.3</td>
<td>1.6</td>
</tr>
<tr>
<td>24 h post-THC</td>
<td>26.4 ± 2.0\textsuperscript{*}</td>
<td>8.4</td>
</tr>
<tr>
<td>7 day post-THC</td>
<td>25.1 ± 3.0\textsuperscript{**}</td>
<td>15.3</td>
</tr>
<tr>
<td>14 day post-THC</td>
<td>30.3 ± 2.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>
minimum effective doses are defined as the lowest dose producing an 0.5 ml/kg increase or decrease outside of the 99% C.I. of saline and were calculated by interpolation of two points.

Descending limb of function was not determined.

\* p<0.05, \** p<0.01 compared to U50,488 control
Table 3. Control ED$_{50}$ values and potency ratios$^a$ at different times after stopping daily THC injections.

<table>
<thead>
<tr>
<th></th>
<th>Potency Ratio</th>
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<tbody>
<tr>
<td></td>
<td>MED</td>
</tr>
<tr>
<td>THC</td>
<td></td>
</tr>
<tr>
<td>Diuresis-ascending</td>
<td>2.2 mg/kg$^b$</td>
</tr>
<tr>
<td>Antinociception</td>
<td>13.2 mg/kg</td>
</tr>
<tr>
<td>AM4054</td>
<td></td>
</tr>
<tr>
<td>Diuresis-ascending</td>
<td>0.05 mg/kg$^b$</td>
</tr>
<tr>
<td>Diuresis-descending</td>
<td>0.54 mg/kg$^b$</td>
</tr>
<tr>
<td>Antinociception</td>
<td>0.3 mg/kg</td>
</tr>
</tbody>
</table>

$^a$ Calculated by dividing the post-THC treatment MED or ED$_{50}$ values by the baseline MED or ED$_{50}$ values.

$^b$ Taken from Table 2; included here for clarity.
Table 4. Urine output (in ml/kg, mean ± SEM) following saline or furosemide before and 24 h after 7-day treatment with 10 mg/kg THC (n=6/group).

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Furosemide</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1ml/100g</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td>Control</td>
<td>14.6 ± 3.6</td>
<td>36.4 ± 3.1</td>
</tr>
<tr>
<td>24 h after THC</td>
<td>18.5 ± 2.6</td>
<td>34.3 ± 3.1</td>
</tr>
</tbody>
</table>
Figure 1
Figure 3
Figure 4
Figure 5