

Cannabinoid Agonists but not Inhibitors of Endogenous Cannabinoid Transport or Metabolism Enhance the Reinforcing Efficacy of Heroin in Rats

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Accumulating evidence suggests that the endogenous cannabinoid system is involved in the reinforcing effects of heroin. In rats intravenously self-administering heroin, we investigated effects of cannabinoid CB₁ receptor agonists and compounds that block transport or metabolism of the endogenous cannabinoid anandamide. The natural cannabinoid CB₁ receptor agonist delta-9-tetrahydrocannabinol (THC, 0.3–3 mg/kg i.p.) did not alter self-administration of heroin under a fixed-ratio one (FR1) schedule, except at a high 3 mg/kg dose which decreased heroin self-administration. Under a progressive-ratio schedule, however, THC dose-dependently increased the number of 50 µg/kg heroin injections self-administered per session and the maximal ratio completed (break-point), with peak increases at 1 mg/kg THC. In addition, 1 mg/kg THC increased break-points and injections self-administered over a wide range of heroin injection doses (25–100 µg/kg), indicating an increase in heroin's reinforcing efficacy and not its potency. The synthetic cannabinoid CB₁ receptor agonist WIN55,212-2 (0.3–3 mg/kg i.p.) had effects similar to THC under the progressive-ratio schedule. In contrast, AM-404 (1–10 mg/kg i.p.), an inhibitor of transport of anandamide, and URB-597 (0.01–0.3 mg/kg i.p.), an inhibitor of the enzyme fatty acid amide hydrolase (FAAH) that degrades anandamide, or their combination, did not increase reinforcing efficacy of heroin at any dose tested. Thus, activation of cannabinoid CB₁ receptors facilitates the reinforcing efficacy of heroin and this appears to be mediated by interactions between cannabinoid CB₁ receptors and mu-opioid receptors and their signaling pathways, rather than by an opioid-induced release of endogenous cannabinoids.

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INTRODUCTION

It has been repeatedly demonstrated that treatment with the cannabinoid CB₁ receptor antagonist Rimonabant (SR-141716A) can decrease the reinforcing effects of heroin (Navarro *et al*, 2001; Caille and Parsons, 2003; De Vries *et al*, 2003; Solinas *et al*, 2003). In addition, mice lacking cannabinoid CB₁ receptors are less sensitive to the

reinforcing effects of morphine (Ledent *et al*, 1999; Martin *et al*, 2000; Cossu *et al*, 2001), and in these mice morphine does not increase dopamine levels in the nucleus accumbens (Mascia *et al*, 1999). These findings strongly support the hypothesis that the endogenous cannabinoid system plays an important role in the reinforcing effects of opioids.

A possible mechanism underlying these effects is that activation of opioid receptors by heroin or morphine results in the release of endogenous cannabinoids and, consequently, in the activation of cannabinoid CB₁ receptors. A similar mechanism has been proposed for the reduction of the reinforcing effects of delta-9-tetrahydrocannabinol (THC), the major psychoactive ingredient in marijuana, by opioid antagonists (Tanda and Goldberg, 2003; Justinova *et al*, 2004; Solinas and Goldberg, 2004; Solinas *et al*, 2004b). Systemic administration of THC, in fact, increases extracellular levels of the endogenous opioid receptor ligand

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beta-endorphin in both the ventral tegmental area (VTA) and the nucleus accumbens, and injection of beta-endorphin into the VTA markedly enhances the ability of rats to discriminate the effects of experimenter-administered THC (Solinas *et al*, 2004b). In addition, it has been shown that cannabinoid CB₁ receptors and mu- and delta-opioid receptors in the striatum are strongly linked at the intracellular level (Berrendero *et al*, 2003). Thus, on one hand, cannabinoid receptor agonists can release endogenous opioids, and, on the other hand, stimulation of opioid receptors can facilitate the signal transduction of cannabinoid receptors. Therefore, it is likely that opioid agonists facilitate certain effects of THC by mimicking some of the effects of endogenous opioids released by THC and, at the same time, facilitating cannabinoid CB₁ receptor-induced effects at the intracellular level. Conversely, the reduction of THC's effects by opioid antagonists is likely due to blockade of the effects of endogenous opioids released by THC and antagonism of CB₁ receptor-induced effects at the intracellular level.

Of the known endogenous ligands for cannabinoid CB₁ receptors, anandamide is one of the best characterized (Freund *et al*, 2003; Piomelli, 2003; Di Marzo *et al*, 2004). It is synthesized on demand, binds with a high affinity to cannabinoid CB₁ receptors and is rapidly inactivated by a two-step mechanism consisting of uptake into cells by a membrane transporter (Di Marzo *et al*, 1994; Beltramo *et al*, 1997; Fegley *et al*, 2004, 2005; Ortega-Gutierrez *et al*, 2004) where it is then degraded by the enzyme fatty acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2002; Fegley *et al*, 2004, 2005). Pharmacological tools are available to block these two steps in the inactivation of endogenously released anandamide (Piomelli, 2003). AM-404 inhibits the transport of anandamide into cells where it is metabolized by FAAH (Piomelli *et al*, 1999) and URB-597 inhibits intracellular FAAH activity (Kathuria *et al*, 2003; Fegley *et al*, 2004, 2005). Administration of these compounds or genetic ablation of the FAAH enzyme results in increased levels of anandamide in brain tissue and peripheral blood (Giuffrida *et al*, 2000; Kathuria *et al*, 2003; Fegley *et al*, 2005) and in the potentiation and prolongation of anandamide's effects (Calignano *et al*, 1997a; Cravatt *et al*, 2001; Kathuria *et al*, 2003).

In this study, we further explored the involvement of the endogenous cannabinoid system in the reinforcing effects of opioids by measuring the effects of directly acting cannabinoid CB₁ receptor agonists and indirectly acting inhibitors of endogenous cannabinoid transport into cells and intracellular FAAH metabolism on heroin self-administration under two different schedules of intravenous drug injection. These two schedules, fixed-ratio and progressive-ratio schedules of drug injection, are differentially sensitive to specific pharmacological, environmental, or surgical manipulations and probably measure different aspects of drug reinforcement processes (Arnold and Roberts, 1997; Solinas *et al*, 2003, 2004a). Under a one-response fixed-ratio (FR1) schedule of drug self-administration, only a single response is required for each drug injection. FR1 schedules are typically used to study levels and patterns of drug intake. However, it may be difficult to determine whether changes in self-administration responding under FR1 schedules are due to increases or decreases in the reinforcing efficacy of the self-administered drug (Arnold

and Roberts, 1997). This difficulty can be overcome by comparing the effects of pharmacological treatments under simple FR schedules and under more complex procedures such as progressive-ratio schedules (Arnold and Roberts, 1997). With progressive-ratio schedules, the number of responses the subject must make in order to obtain each successive drug injection progressively increases within a session until the subject stops responding. This end point, generally referred to as a 'break point', is believed to be a direct measure of the motivational strength of the reinforcing event (Hodos, 1961). Thus, progressive-ratio schedules can provide important information on the role the endogenous cannabinoid system plays in facilitating the reinforcing effects of heroin.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats (Charles River, Wilmington, Mass., USA) experimentally naive at the start of the study and initially weighing 300–350 g were used for all experiments. All rats were housed individually in a temperature- and humidity-controlled room and were maintained on a 12-h light/dark cycle; the lights were on from 0645 to 1845 hours. Experiments were conducted during the light phase. Rats had free access to food until recovery from surgery for implantation of i.v. catheters. One day before the start of daily self-administration sessions, food was restricted to approximately 15 g/day. Food was always given shortly after the end of daily experimental sessions. Water was available *ad libitum* for all animals. Animals used in this study were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and all experiments were conducted in accordance with the guidelines of the Institutional Care and Use Committee of the Intramural Research Program, National Institute on Drug Abuse (NIDA), National Institutes of Health and the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2003).

Catheterization

Catheters were implanted in the right jugular vein under aseptic conditions using i.p. ketamine (60 mg/kg) and xylazine (10 mg/kg) anesthesia. Catheters consisted of approximately 4 cm of Silastic tubing (0.044 mm ID, 0.814 mm OD) connected to vinyl tubing (Dural Plastics, 0.5 mm ID, 1.0 mm OD) with a 23-g stainless-steel tube and bonded with polyethylene shrink tubing. A 10-mm long section of Tygon tubing (5 mm OD) was glued to the catheter at the midpoint of the vinyl tubing and used as a subcutaneous anchor when the catheter exited the skin in the neck. The right external jugular vein was exposed by blunt dissection and the silastic portion of the catheter was inserted into the vein and sutured into place. The vinyl portion of the catheter was passed subcutaneously to the back of the neck, where the tip exited and was obturated with a modified 23-g needle. The incision was closed with stainless-steel wound clips. A 20 mm plastic bolt was cemented with dental acrylic to four stainless-steel jeweler's

screws implanted in the skull. During experimental sessions, the catheter was connected to the injection pump via tubing passing through a metal spring that was attached to the plastic bolt on the rats' head. Catheters were flushed before and after each session with 0.1 ml of saline solution.

Heroin Self-Administration Apparatus and Procedure

Experimental chambers (30 × 24 × 29 cm, Coulbourn Instruments, Allentown, Pa., USA) were enclosed individually in sound-attenuation chests. Each experimental chamber had a metal grid floor, a recessed food tray, and two nose-poke holes in the right wall. A 4500-Hz auditory stimulus was provided by a Sonalert (model #628) operated at 8.75 V. Illumination was provided by a shielded white light bulb (type #1820) at all times except during injection and time-out periods, when both the tone and the light were pulsed at a rate of 5 Hz, with one stimulus on while the other was off. Drug solution was delivered through Tygon tubing, protected by a metal spring and suspended through the ceiling of the experimental chamber from a single-channel fluid swivel. This tubing was attached to a syringe pump (Med Associates, St Albans, Vt., USA) that delivered fluid at a rate of 3.19 ml/min using a 10-ml syringe. Unit doses were manipulated by computerized adjustment of the duration of the injection (0.5–2 s). Experimental events were controlled by microcomputers using Med Associates interface and software (Med Associates Inc., East Fairfield, Vt., USA).

Heroin self-administration under a FR1 schedule. Self-administration sessions began approximately 1 week after catheter implantation. A single response in the left nose-poke hole immediately activated the injection pump and also caused the house light to flash and a tone to pulse for 5 s. During this 5-s time-out period, responding had no programmed consequences. Following the time-out, the next response in the left hole was reinforced. Responses in the nose-poke hole on the right side were recorded but never reinforced. Sessions lasted 3 h. During initial training, a unit dose of 100 µg/kg/injection heroin was made available under the FR1 schedule described above. All rats began self-administering heroin within a few sessions. When consistent self-administration responding was obtained, the dose of heroin was reduced to 50 µg/kg/injection and sessions were run until a stable baseline was reached. Baseline responding was considered stable when the number of injections per session did not differ more than 10% for three consecutive days. After stabilization rats were either tested with THC or moved to the progressive-ratio schedule.

Heroin self-administration under a progressive-ratio schedule. Rats first learned to respond for heroin under a FR1 schedule as described above and then were switched to the progressive-ratio schedule. Under the progressive-ratio schedule of i.v. drug injection, the response requirement increased with each successive injection. The steps of the exponential progression were the same as developed by Roberts and Bennett (1993) and previously used by us (Solinas *et al*, 2003, 2004a), based on the equation: response ratio = $(5 \times e^{(0.2 \times \text{infusion number})}) - 5$, rounded to the nearest integer. Thus, the values of the steps were 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402,

492, 603, and 737. Sessions under the progressive-ratio schedule lasted 5 h or until 30 min passed without a response. The breaking point was defined as the step number of the last ratio completed before 30 min of nonresponse or at the end of the 5 h.

Within three to five sessions, rats reached stability under the progressive-ratio schedule. Baseline responding was considered stable when the number of injections per session did not differ more than 10% for three consecutive days. Rats were then either tested with different doses of cannabinoid compounds administered in a random order or switched to a different dose of heroin. Each compound was tested on one group only. Between test sessions, there were at least two control sessions in order to re-establish baseline responding. Different injection doses of heroin for self-administration were made available in descending order (100, 50, 25, 12.5 µg/kg/injection).

Drugs

Heroin (National Institute on Drug Abuse, Baltimore, MD, USA) was dissolved in distilled water. Delta-9-THC (National Institute on Drug Abuse) 50 mg/ml in ethanol was dissolved in a solution 40% w/v of cyclodextrin (RBI/SIGMA, Natick, MA, USA). WIN55,212-2 (RBI-Sigma) was suspended in 0.3% Tween-80 in saline and administered i.p. in a volume of 2 ml/kg. AM-404 (synthesized at the University of Connecticut, Storrs, CT, USA) was suspended in 0.3% Tween-80 in saline and administered i.p. in a volume of 2 ml/kg 60 min before the start of the session. URB-597 was synthesized as previously described (Mor *et al*, 2004) and donated by Drs A Duranti, A Tontini, G Tarzia, and D Piomelli. URB-597 was dissolved in a 50% DMSO/H₂O solution and administered i.p. in a volume of 2 ml/kg 40 min before the start of the session.

Data Analysis

Data for the effects of different doses of THC on FR1 schedules of heroin self-administration were analyzed as number of injections (\pm SEM) per session. Data were subjected to a repeated measures ANOVA, followed by *post hoc* Dunnett's test with comparisons with the baseline condition.

Data for the effects of the 3 mg/kg dose of THC on FR1 schedules of heroin self-administration were further analyzed as number of injections (\pm SEM) per 30-min block of each session. These data were subjected to a repeated measures two-way ANOVA, followed by *post hoc* Student–Newman–Keuls' test. Patterns of responding during these sessions were also analyzed by sorting the postinjection pauses from each session into 1-min bins and displaying them as relative frequency distributions. These data (showing the percentage \pm SEM of postinjection pauses in each bin) were subjected to a repeated measures two-way ANOVA, with paired comparisons performed using the Student–Newman–Keuls' test.

Variability in patterns of responding under the FR1 schedule was analyzed using root mean square of successive differences (rMSSD; for details, see Tornatzky and Miczek, 2000; Panlilio *et al*, 2003). As the mean postinjection pauses differed between baseline responding and responding after

injections of 3 mg/kg of THC, rMSSD was scaled by dividing it by the mean postinjection pause for each rat (see Panlilio *et al*, 2003). Data were subjected to a repeated measures two-way ANOVA, followed by *post hoc* Student–Newman–Keuls' test.

Self-administration data for the effects of THC, WIN55,212-2, AM-404 and URB-597 on progressive-ratio schedules of heroin self-administration were analyzed as last (maximal) ratio completed per session (\pm SEM) after a logarithmic transformation for the progressive-ratio experiments. Data were subjected to a repeated measures ANOVA, followed when appropriate by *post hoc* Dunnett's test with comparisons with the base line condition. For analysis of the effects of THC 1 mg/kg on heroin self-administration over a wide range of heroin doses, data were subjected to a repeated measures two-way ANOVA, followed by *post hoc* Student–Newman–Keuls' test. Changes were considered significant when $P < 0.05$.

RESULTS

Effects of THC on Heroin Self-Administration under a FR1 Schedule

Systemic administration of THC (0.3–3 mg/kg *i.p.*) did not increase the number of self-administered heroin injections under the FR1 schedule (Figure 1). In contrast, at the 3 mg/kg dose, THC significantly reduced the number of self-administered heroin injections (Figure 1) ($F(3,18) = 4.849$, $P < 0.05$). It is unlikely that this reduction was due to nonselective direct depressant effects of THC on behavior since THC at this dose does not decrease locomotor activity in rats (Sanudo-Pena *et al*, 2000). In addition, when we compared the number of heroin injections rats self-administered during different phases of the experimental session (30-min blocks), numbers of self-administered heroin injections during the first and second 30-min intervals of the session were similar during baseline conditions and after 3 mg/kg of THC (Figure 2a), when the depressant effects of THC (as measured by depression of rates of food-maintained responding in a THC drug discrimination procedure (Solinas and Goldberg, personal observations) should be maximal. Rather, maximal decreases in the number of heroin injections self-administered (which did not reach statistical significance) were found later in the session. Self-administration behavior returned toward baseline levels during the last 30-min interval of the session when the rate-depressant effects of THC may have been diminishing. In addition, reduction of heroin self-administration did not appear related to an antagonism of heroin's reinforcing effects, since the pattern of self-administration responding after 3 mg/kg THC did not resemble that during extinction (a number of self-administered injections at the start of the session followed by almost complete cessation of responding and injections for the remainder of the session (see Ettenberg *et al*, 1982, for example). Visual inspection of event records (see Figure 2b and c, for a representative sample) indicated that responding was very regular under baseline conditions (Figure 2b) but became irregular ('dysregulated') after administration of 3 mg/kg of THC (Figure 2c), with rats tending to self-administer injections of heroin in short bursts separated by

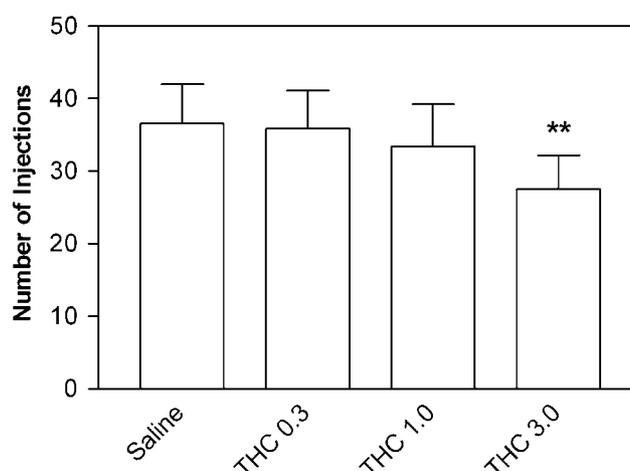


Figure 1 Effects of different doses of THC on self-administration of 50 μ g/kg injections of heroin under a fixed-ratio 1 (FR1) schedule. Data are expressed as mean (\pm SEM) number of injections per session during baseline (BL) conditions and during sessions with THC pretreatment. **, $P < 0.01$. *Post hoc* comparison of BL vs treatment session after significant ANOVA for repeated measures main effect, Dunnett's test ($n = 7$).

considerable amounts of time. Relative frequency distributions of mean postinjection pauses in responding (expressed as percentage of total injections obtained in a session) are shown in Figure 2d and confirm that THC injections produced a significant increase in the number of heroin injections that were self-administered after very short (1 min or less) or very long intervals of time (> 15 min) ($F(1,15) = 65.21$, $P < 0.0001$). In order to determine whether or not THC significantly changed the pattern of heroin self-administration behavior, rMSSD, a measure of the within-subject variability of sequential postinjection pauses, were determined for each rat self-administering heroin under baseline conditions (baseline sessions before and after the test session, BL1 and BL2) and after treatment with the 3 mg/kg dose of THC. The absolute levels of variability (data not shown), as well as the relative variability (Figure 2e), were significantly greater after THC administration compared to these baseline levels ($F(2, 12) = 20.245$, $P < 0.0001$ and $F(2,12) = 18.264$, $P < 0.001$, respectively).

In order to determine whether or not lower doses of THC altered the pattern of heroin self-administration during the session in ways that would not be reflected in whole session means for self-administered injections, the pattern of heroin self-administration during the 3-h sessions (number of injections during each 30-min block of the session) and the distribution of mean postinjection pauses (rMSSD) were analyzed and event records were visually inspected at these doses. We found that administration of 0.3 and 1 mg/kg doses of THC did not produce any change in the patterns of heroin self-administration compared to baseline levels (data not shown), confirming our finding that only a high 3 mg/kg dose of THC alters heroin self-administration under a FR1 schedule. Thus, under a FR1 schedule of intravenous heroin self-administration, a high, but not a low or intermediate, dose of THC reduced the number of injections self-administered per session, but this decrease could not be easily interpreted as a decrease or an increase in the reinforcing efficacy of heroin. Given the difficulties in

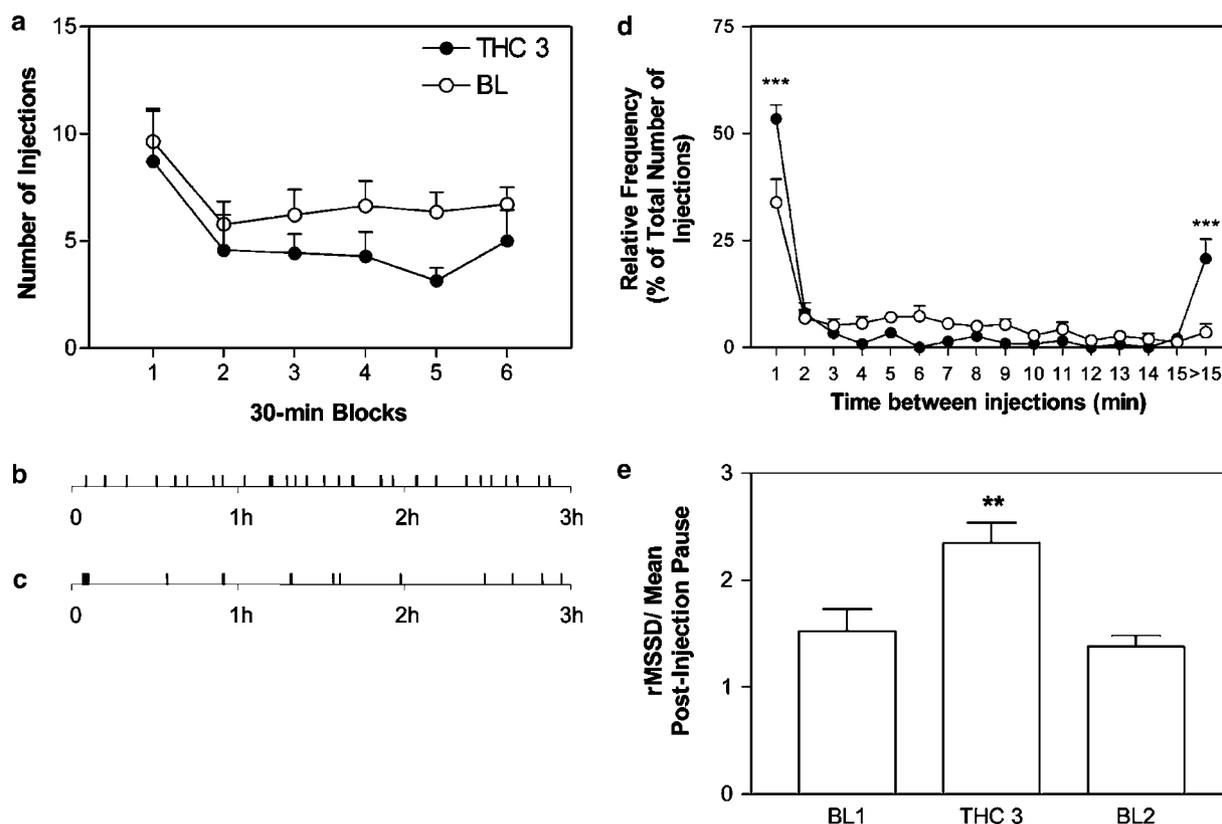


Figure 2 Effects of pretreatment with 3 mg/kg of THC on the pattern of self-administration of 50 µg/kg injections of heroin under a fixed-ratio 1 (FR1) schedule. Data in (a) are expressed as mean (\pm SEM) number of injections in six consecutive 30-min blocks for a total 3 h session, during baseline conditions (empty symbols) and during the session with THC pretreatment (filled symbols). Representative event records of one rat under baseline (BL) conditions (b) and during the session with THC pretreatment (c) are shown. Data in (d) are expressed as mean (\pm SEM) of the percentage of heroin injections that occurred after each indicated postreinforcement time expressed in minutes during baseline conditions (empty symbols) and during the session with THC pretreatment (filled symbols). Data in (e) are expressed as mean (\pm SEM) of the within-subject rMSSD of latencies in sec between consecutive injections of heroin scaled by mean latency during baseline conditions (BL1 and BL2) and during the session with THC pretreatment. Note that BL1 represents the baseline condition before and BL2 the baseline condition after the test (THC 3) session. **, $P < 0.05$; ***, $P < 0.001$. *Post hoc* comparison with vehicle controls after significant two-way ANOVA main effect, Student–Newman–Keuls' test ($n = 7$).

interpreting the effects of THC on self-administration of heroin under FR1 schedules, we decided to investigate the effects of THC on heroin self-administration under a progressive-ratio schedule.

Effects of THC on Heroin Self-Administration under a Progressive-Ratio Schedule

Systemic administration of THC significantly and dose-dependently increased the break point for self-administration of a 50 µg/kg injection dose of heroin under the progressive-ratio schedule, as measured by number of injections in the session and by the corresponding last ratio completed (Figure 3a) ($F(5,15) = 5.421$, $P < 0.01$). An i.p. dose of 1 mg/kg THC produced the peak effect, while a higher 3 mg/kg dose of THC increased break points in some rats but completely disrupted responding in other rats, resulting in an inverted U-shaped dose-response curve (Figure 3a).

When heroin injection dose was varied, the 1 mg/kg dose of THC significantly increased the number of injections per session and the corresponding last ratio completed (Figure 3b) over a wide range of heroin doses (Dose, $F(6,18) = 5.588$, $P < 0.01$; Treatment, $F(1,6) = 16.205$, $P < 0.01$), resulting in

upward shifts, rather than left-ward shifts, in the dose-response curves for heroin self-administration. Thus, THC pretreatment appeared to increase the reinforcing efficacy rather than the potency of heroin.

Representative cumulative-response records are shown in Figure 4 for the effects of 1 mg/kg THC on self-administration of several injection doses of heroin. THC increased responding for self-administration of all heroin injection doses studied, with the exception of the highest 100 µg/kg injection dose of heroin. At this dose of heroin, any increase in heroin's reinforcing efficacy may have been masked by direct depressant effects of heroin or the combination of heroin and THC.

Effects of WIN55,212-2 on Heroin Self-Administration under a Progressive-Ratio Schedule

Systemic administration of the synthetic CB1 agonist WIN55,212-2 significantly and dose-dependently increased the break point for self-administration of a 50 µg/kg injection dose of heroin under the progressive-ratio schedule, as measured by number of injections in the session and by the corresponding last ratio completed (Figure 5) ($F(3,21) = 5.473$, $P < 0.01$). Consistent with

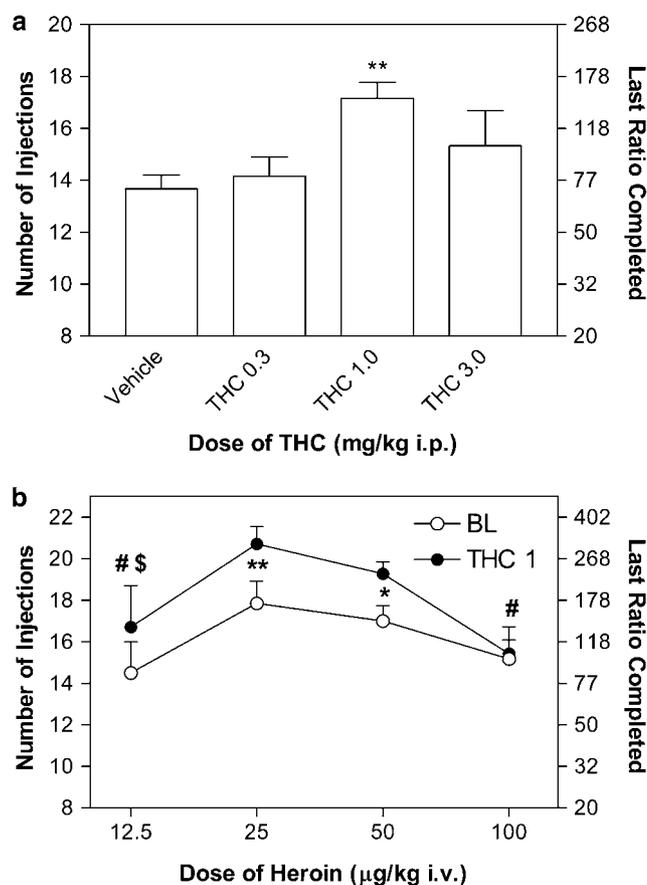


Figure 3 Effects of THC pretreatment on self-administration of heroin under a progressive-ratio schedule. Data are expressed as mean (\pm SEM) number of injections per session (left ordinates) and the corresponding last ratios completed (right ordinates) during baseline (BL) conditions and during sessions with THC pretreatment. Panel (a) shows the effects of different doses of THC on self-administration of a fixed dose (50 μ g/kg/injection) of heroin. **, $P < 0.01$. *Post hoc* comparison of BL vs treatment session after significant ANOVA for repeated measures main effect, Dunnett's test ($n = 6$). Panel (b) shows the effects of pretreatment with a single 1 mg/kg dose of THC on self-administration of different doses (12.5–100 μ g/kg/injection) of heroin. *, $P < 0.05$; **, $P < 0.01$ indicate differences between baseline vs treatment session. #, $P < 0.05$ indicates differences from the heroin dose of 25 μ g/kg/injection. \$, $P < 0.05$ indicates differences from the heroin dose of 50 μ g/kg/injection. Two-way ANOVA for repeated measures main effect, Student–Newman–Keuls' test ($n = 7$).

previous studies (Wiley *et al*, 1995), the potency of WIN55,212-2 was similar to that of THC, with a peak effect at an i.p. dose of 1 mg/mg.

Effects of Compounds that Inhibit Transport into Cells or Intracellular Metabolism of Endogenous Cannabinoids on Heroin Self-Administration under a Progressive-Ratio Schedule

Systemic administration of AM-404 or URB-597 can increase blood and brain levels of the endogenous cannabinoid anandamide (Giuffrida *et al*, 2000; Kathuria *et al*, 2003; Fegley *et al*, 2005) and should increase the duration of action of any anandamide released by heroin. Systemic administration of AM-404 (1–10 mg/kg given i.p. 60 min before the session) or URB-597 (0.01–0.3 mg/kg given i.p. 40 min before the session) did not increase the

break point for heroin self-administration under the progressive-ratio schedule, as measured by number of heroin injections self-administered per session or the corresponding last ratio completed (Figure 6a and b) at any dose of AM-404 or URB-597 tested. To the contrary, at the highest dose tested, AM-404 (10 mg/kg) significantly decreased the break point for heroin self-administration ($F(3,27) = 3.668$, $P < 0.05$) without significantly affecting rates of nose-poke responding in the inactive hole (data not shown).

Results were similar when the 3 mg/kg dose of AM-404 (the highest dose of AM-404 that did not decrease heroin self-administration) was coadministered with different doses of URB-597 (0.01–0.3 mg/kg). There was no increase in the number of heroin injections self-administered or the corresponding last ratio completed (Figure 7). However, coadministration of 3 mg/kg AM-404 and 0.1 or 0.3 mg/kg URB-597, which by themselves did not alter the number of heroin injections self-administered per session or the last ratio completed, significantly decreased these values when given together (Figure 7) ($F(4,28) = 3.008$, $P < 0.05$) without significantly affecting rates of nose-poke responding in the inactive hole (data not shown).

DISCUSSION

The reinforcing efficacy of heroin measured under the progressive-ratio schedule of intravenous heroin self-administration was significantly enhanced by THC administration. The increase in heroin's reinforcing efficacy produced by THC resulted in an upward shift in the full heroin dose–effect function. This indicates that combinations of THC and heroin produced synergistic effects that were greater than those of heroin alone. WIN55,212-2, a synthetic compound CB₁ receptor agonist that activates CB₁ receptors with a potency similar to THC *in vivo* (Wiley *et al*, 1995), also enhanced the reinforcing efficacy of heroin under the progressive-ratio schedule. The similar effects of THC and WIN55,212-2 in increasing break points for intravenous self-administration of heroin under the progressive-ratio schedule provide additional evidence that activation of cannabinoid CB₁ receptors can increase the reinforcing efficacy of opioids. In previous studies, we and others further demonstrated that the reinforcing effects of heroin in rats are reduced by the cannabinoid CB₁ receptor antagonist rimonabant (SR-141716A) (Navarro *et al*, 2001; Caille and Parsons, 2003; De Vries *et al*, 2003; Solinas *et al*, 2003). In addition, genetic ablation of cannabinoid CB₁ receptors decreases the rewarding effects of opiates (Ledent *et al*, 1999; Mascia *et al*, 1999; Martin *et al*, 2000). The present findings that the reinforcing effects of heroin are enhanced by cannabinoid CB₁ receptor agonists extend these previous findings and clearly demonstrate an important facilitatory role for the cannabinoid system in the reinforcing effects of opioids.

Although the reinforcing efficacy of heroin was significantly enhanced under the progressive-ratio schedule at an intermediate 1 mg/kg dose of THC in the present study, a higher 3 mg/kg dose of THC no longer produced significant enhancement. This finding is consistent with reported findings of biphasic effects of THC, with low doses having

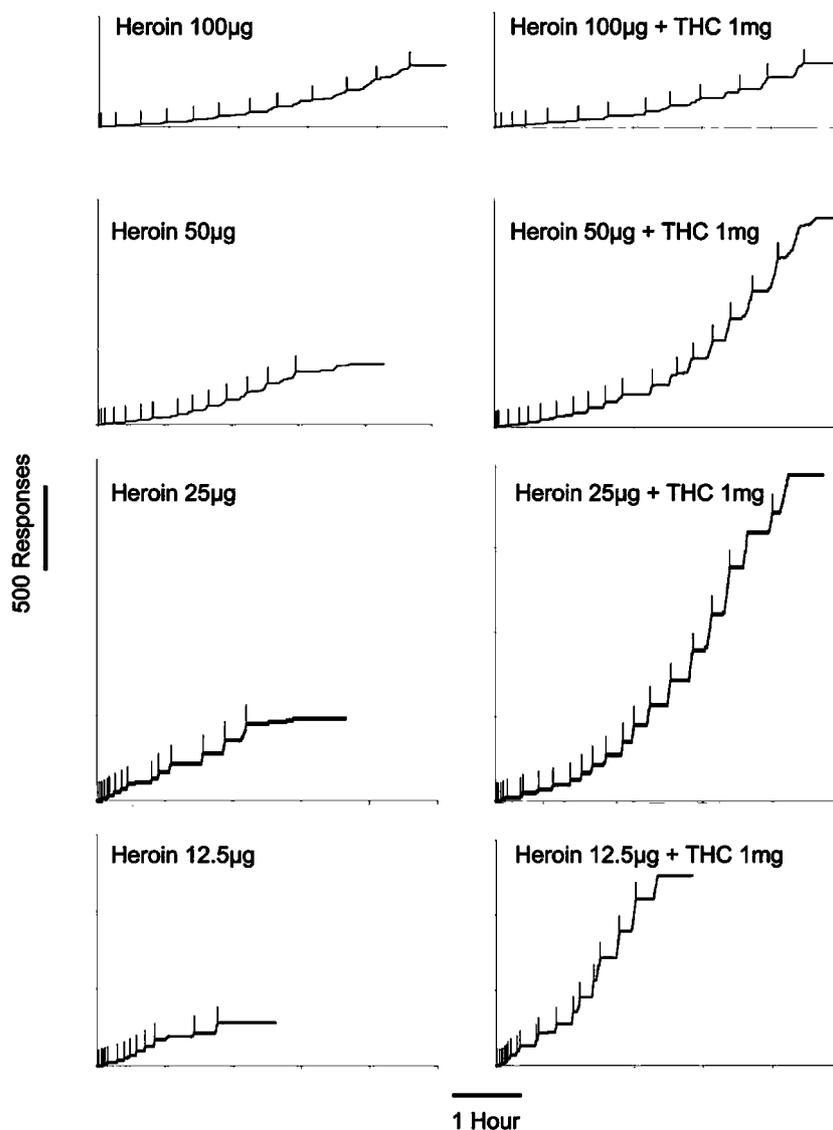


Figure 4 Representative cumulative-response records of heroin self-administration under the progressive-ratio schedule. Ordinates represent cumulative number of nose-poke responses and abscissas represent time. On the left-hand side of the graph, records representative of behavior at different doses of heroin under baseline conditions are shown. At doses of 12.5, 25, 50, and 100 µg/kg/injection, the number of injections of heroin self-administered per session was 13, 15, 14, and 13 and the last ratio completed was 62, 95, 77, and 62, respectively. On the right-hand side of the graph, records representative of behavior at different doses of heroin after pretreatment with THC are shown. After THC pretreatment, the number of injections of heroin self-administered per session at doses of 12.5, 25, 50, and 100 µg/kg/injection was 19, 21, 19, and 13, and the last ratio completed was 219, 328, 219, and 62, respectively. Note that an increase in break point was evident at all doses from 12.5 to 50 µg/kg/injection, but not at the highest 100 µg/kg/injection dose.

activating and reinforcing effects and higher doses having depressant and aversive effects (Lepore *et al*, 1995; Sanudo-Pena *et al*, 2000; Valjent and Maldonado, 2000). Thus, the lack of effect found at the 3 mg/kg dose of THC may be due to depressant effects of THC at this dose or to synergistic depressant effects of experimenter-administered THC and heroin self-administered by the rats. This second possibility is supported by our finding in another study (Solinas and Goldberg, 2005) that examined the effects of THC on responding for food pellets under a progressive-ratio schedule. In that study, a 1 mg/kg dose of THC did not alter responding for food, but 3 mg/kg THC produced peak increases in the break points for food, suggesting that combined effects of heroin and THC, rather than THC's effects alone, were responsible for the failure of 3 mg/kg

THC to increase the break point for heroin self-administration in the present study. Decreases in response output due to THC-heroin combinations could also be responsible for the lack of increases in break points when 1 mg/kg of THC was administered to rats self-administering the high 100 µg/kg injection dose of heroin. Thus, direct depressant effects of 100 µg/kg injections of heroin in combination with a potentially enhancing 1 mg/kg dose of THC may have produced synergistic depression of responding that masked an increase in reinforcing efficacy of heroin.

In contrast to results under the progressive-ratio schedule of heroin self-administration, we only observed THC-induced decreases in heroin self-administration under a FR1 schedule. It is well known that a decrease in heroin self-administration under a FR1 schedule can result from either

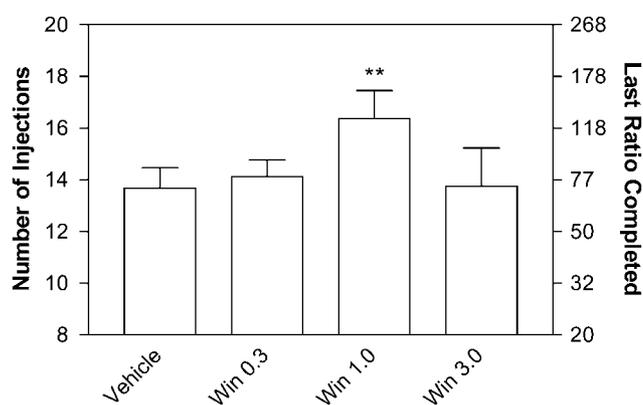


Figure 5 Effects of pretreatment with different doses of WIN55,212-2 on self-administration of 50 µg/kg injections of heroin under a progressive-ratio schedule. Data are expressed as mean (\pm SEM) number of injections per session (left ordinates) and the corresponding last ratios completed (right ordinates) during baseline conditions and during sessions with WIN55,212-2 pretreatment. **, $P < 0.01$. *Post hoc* comparison of BL vs treatment session after significant ANOVA for repeated measures main effect, Dunnett's test ($n = 8$).

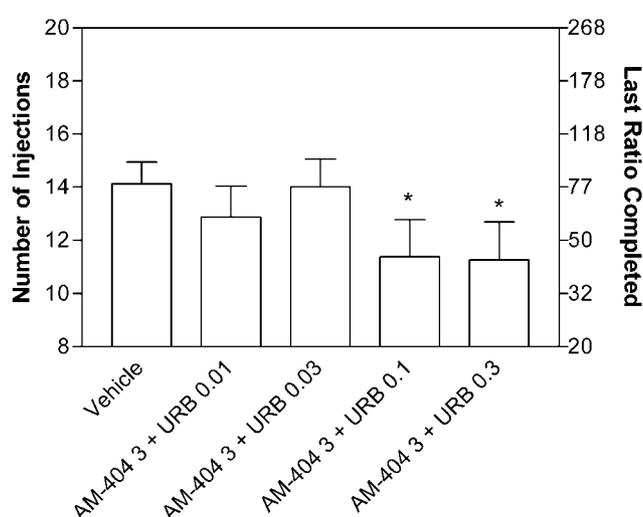


Figure 7 Effects of pretreatment with combinations of AM-404 and URB-597 on self-administration of 50 µg/kg injections of heroin under a progressive-ratio schedule. Data are expressed as mean (\pm SEM) number of injections per session (left ordinates) and the corresponding last ratios completed (right ordinates) during baseline (BL) conditions and during sessions in which combinations of 3 mg/kg of AM-404 and different doses of URB-597 were administered. *, $P < 0.05$. *Post hoc* comparison of BL vs treatment session after significant ANOVA for repeated measures main effect, Dunnett's test ($n = 8$).

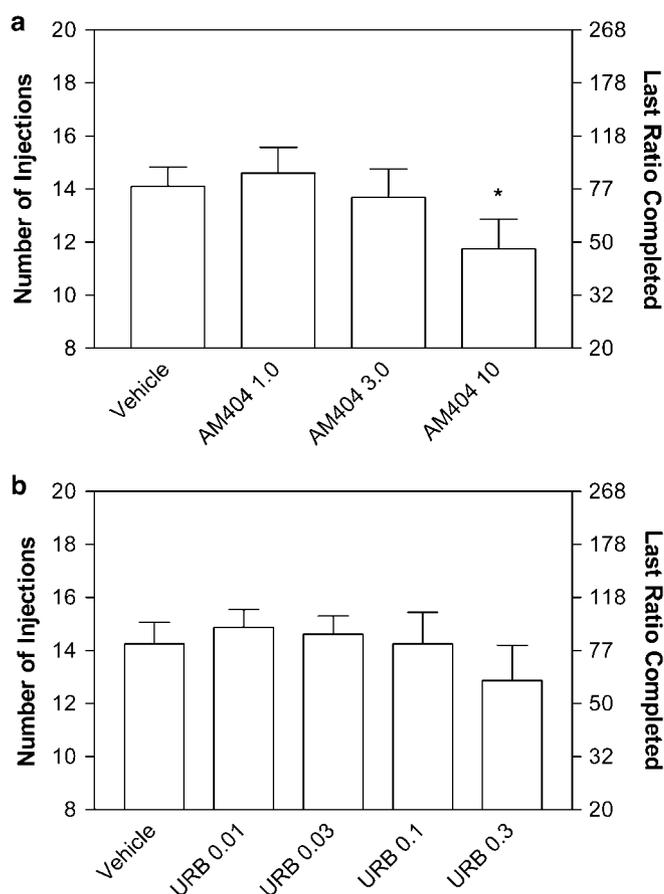


Figure 6 Effects of pretreatment with different doses of AM-404 (a) or URB-597 (b) on self-administration of 50 µg/kg injections of heroin under a progressive-ratio schedule. Data are expressed as mean (\pm SEM) number of injections per session (left ordinates) and corresponding last ratios completed (right ordinates) during baseline (BL) conditions and during sessions with AM-404 ($n = 10$) or URB-597 ($n = 8$) pretreatment. *, $P < 0.05$. *Post hoc* comparison of BL vs treatment session after significant ANOVA for repeated measures main effect, Dunnett's test.

an increase in reinforcing efficacy (eg, when the dose of heroin is increased) or a decrease in reinforcing efficacy (eg, when the dose of heroin is decreased) (Arnold and Roberts, 1997). Thus, a FR1 schedule of drug self-administration is well suited for measuring changes in drug intake, but not for detecting changes in reinforcing efficacy. An in-depth analysis of self-administration behavior under the FR 1 schedule showed that THC-induced reductions in self-administered heroin injections were accompanied by a change in the pattern of self-administered injections within individual sessions. Under baseline conditions, heroin self-administration was relatively regular, but after THC administration heroin self-administration became irregular ('disregulated'), that is, animals tended to self-administer heroin in bursts of several injections followed by relatively long pauses. Burst-like patterns have been observed after long sessions of cocaine self-administration (Ahmed and Koob, 1998; Tornatzky and Miczek, 2000) and the question has been raised whether such patterns were due to a 'local tolerance or sensitization', that is, a short-lasting decrease or increase, respectively, in the reinforcing efficacy of the drug (Koob *et al*, 1998). Although these THC-induced decreases in heroin intake under the FR1 schedule may be interpreted as consistent with our findings that THC and other directly-acting cannabinoid CB₁ agonists can increase the reinforcing efficacy of heroin under the progressive-ratio schedule, the burst-like patterns of self-administration behavior observed under the FR1 schedule after THC administration do not necessarily reflect 'local' changes in reinforcing efficacy of heroin. Variability in response patterns for drug self-administration is influenced by many factors other than reinforcing efficacy (Panlilio *et al*, 2003). For example, regulated drug intake depends heavily on pharmacokinetics and the detection of drug levels by the animal (Tsibulsky and Norman, 1999). High doses of THC

may have altered the discriminative stimulus effects of heroin under the FR1 schedule or in some other way disrupted the rats' ability to detect the effects of the self-administered heroin. The present results demonstrate once more the difficulties inherent in interpreting changes in response rates under FR1 schedules of drug injection as changes in reinforcing efficacy and the necessity of comparing the influences of experimental manipulations on different schedules of reinforcement and analyzing patterns of responding in addition to rates of responding.

Acute administration of opioid agonists such as morphine has been shown to increase anandamide levels in brain areas such as the nucleus accumbens, the caudate putamen, and the hippocampus (Vigano *et al*, 2004). If heroin self-administration in this study released endogenous cannabinoids such as anandamide, brain levels of anandamide that normally are removed from the extracellular space and degraded very quickly would be increased by inhibiting transport into cells with AM-404 or inhibiting intracellular FAAH metabolism with URB-597 and this should lead to an increased activation of cannabinoid CB₁ receptors similar to that produced by exogenous administration of either THC or WIN55,212-2. Behavioral testing in the present study was well within the range of doses of AM-404 and URB-597 which have been shown to produce substantial elevations in anandamide levels and enhance the effects of exogenously administered anandamide in earlier studies (Giuffrida *et al*, 2000; Kathuria *et al*, 2003; Fegley *et al*, 2004). The fact that neither AM-404 nor URB-597 nor their combination increased the reinforcing effects of heroin under the progressive-ratio schedule suggests that either (1) opioids do not release anandamide in brain areas mediating reinforcement processes; (2) increases in endogenous levels of anandamide produced by opioids are not sufficient to activate cannabinoid CB₁ receptors in brain areas mediating reinforcement processes; or (3) URB-597 and AM-404 have secondary effects not mediated by cannabinoid CB₁ receptors that counteract and mask the effects of any endogenous cannabinoids that are released.

Although AM-404 or combinations of AM-404 and URB-597, at the highest doses administered, significantly reduced break points for heroin self-administration, it is unlikely that this decrease reflects a decrease in the reinforcing efficacy for heroin. In our opinion, two possible mechanisms can underlie this decrease: (1) an enhancement of the effects of released endogenous cannabinoid in brain areas involved in motor behavior may have produced depressant effects on behavior not specifically related to reinforcement of heroin self-administration behavior or (2) the decrease found at higher doses of these compounds may be related to nonspecific depressant effects produced by the drugs alone or by their combination with the effects of self-administered heroin. These depressant effects would have affected high rates of responding on the active nose-pokes, whereas low rates of responding on the inactive lever were not affected.

At the doses tested in this study, the behavioral effects of AM-404 *in vivo* appear to be primarily mediated by inhibition of anandamide transport into cells and the consequent elevation in extracellular levels of endogenous cannabinoids in the brain (Calignano *et al*, 1997a,b; Beltramo *et al*, 2000; Fernandez-Espejo *et al*, 2004; Chhatwal *et al*, 2005; Rodella *et al*, 2005). In contrast, in some *in vitro*

experiments AM-404 activated vanilloid TRPV1 type receptors at concentrations similar to or lower than those necessary for inhibition of anandamide transport (De Petrocellis *et al*, 2000; Zygmunt *et al*, 2000; Ross *et al*, 2001). In addition, at the doses tested in this study, AM-404 and URB-597 increase brain levels of anandamide, which itself activates vanilloid TRPV1 type receptors (Zygmunt *et al*, 1999; Di Marzo *et al*, 2001). Vanilloid TRPV1 receptors are expressed in the brain and, particularly, in areas rich in dopaminergic cell bodies (Mezey *et al*, 2000), and it has been reported that their activation can lead to a release of glutamate in the VTA and substantia nigra and increased dopaminergic firing (Marinelli *et al*, 2003, 2004). Since inhibition of glutamate release in the VTA is believed to be responsible for cannabinoid CB₁ receptor-induced increases in dopaminergic firing and elevations of dopamine in the nucleus accumbens (Szabo *et al*, 2002; Melis *et al*, 2004), activation of CB₁ receptors by elevated anandamide levels and activation of TRPV1 receptors could have opposite effects. However, since both CB₁ and TRPV1 activation results in increased dopaminergic firing (French *et al*, 1997; Marinelli *et al*, 2004), it is likely that the glutamatergic terminals on which cannabinoid CB₁ and vanilloid TRPV1 receptors act are different, with CB₁-responsive terminals making synapses on GABAergic terminals and TRPV1-responsive terminals making synapses directly on dopaminergic neurons. Thus, activation of cannabinoid CB₁ and vanilloid TRPV1 receptors would produce similar and possibly additive effects. The possibility that the decreases in break point for heroin self-administration produced by AM-404 alone or combinations of AM-404 and URB-597 were due, in whole or in part, to activation of vanilloid TRPV1 receptors deserves future investigation. Nonetheless, the fact that URB-597 did not potentiate the reinforcing effects of heroin at doses that increase anandamide levels in the brain indicates that elevations in anandamide levels do not play a role in the modulation of the reinforcing effects of heroin.

Taken together, the present findings that the reinforcing effects of heroin can be enhanced by 'direct' agonists at cannabinoid CB₁ receptors but not by compounds that increase levels of endogenous cannabinoid CB₁ receptor agonists, along with previous findings that CB₁ receptor blockade or genetic deletion of CB₁ receptors reduces the reinforcing effects of opioids (Ledent *et al*, 1999; Mascia *et al*, 1999; Martin *et al*, 2000; Cossu *et al*, 2001; Navarro *et al*, 2001; Caille and Parsons, 2003; De Vries *et al*, 2003; Solinas *et al*, 2003), indicate that the modulation of the reinforcing effects of heroin produced by cannabinoid CB₁ receptor activation or blockade or by genetic deletion of CB₁ receptors is due to interactions between opioid and cannabinoid receptors and their signaling pathways and not to an opioid-induced release of endogenous cannabinoids. Thus, in the absence of cannabinoid CB₁ receptors or in conditions of pharmacological blockade of CB₁ receptors, the signaling strength of mu-opioid receptors would be decreased and, conversely, with cannabinoid CB₁ receptor stimulation the signaling strength of mu-opioid receptors would be increased. In a parallel manner, it has been recently demonstrated that the signaling strength of cannabinoid CB₁ receptors, as measured by increased (³⁵S)-GTPγS binding, is significantly reduced in mice

lacking both mu- and delta-opioid receptors (Berrendero *et al*, 2003). The facts that cannabinoid CB₁ receptors and mu-opioid receptors have similar anatomical distributions in the brain (Matsuda *et al*, 1990; Herkenham *et al*, 1991; Rodriguez *et al*, 2001), that both cannabinoid CB₁ receptors and mu-opioid receptors are members of the G-protein-coupled family of receptors, and that they both modulate similar transduction systems (Reisine *et al*, 1996; Howlett, 2002) support the hypothesis that the reduction in the reinforcing effects of opioids produced by cannabinoid CB₁ receptor activation and the enhancement of the reinforcing effects of opioids produced by cannabinoid CB₁ receptor blockade or deletion are due to interactions between opioid and cannabinoid receptors and their signaling pathways and not to an opioid-induced release of endogenous cannabinoids.

In a previous study, we found that a history of THC exposure did not increase the likelihood that rats would subsequently acquire heroin self-administration under a FR1 schedule and did not alter the number of injections per session or break point values under a progressive-ratio schedule (Solinas *et al*, 2004a). In that study, all THC exposure occurred several days prior to any heroin exposure. In contrast, in the present study, all rats acquired heroin self-administration prior to being tested with THC or WIN55,212-2 and the effects of these cannabinoid CB₁ agonists were assessed while the rats were self-administering heroin. These two studies, together, suggest that previous exposure to THC does not alter the reinforcing efficacy of heroin, but simultaneous exposure to THC and heroin may enhance the reinforcing efficacy of heroin. However, neither study assessed the possibility that simultaneous exposure to THC and heroin may alter the likelihood of acquiring heroin self-administration or increase the likelihood of dependence developing in 'casual' heroin users. We are not aware of epidemiological or laboratory-based attempts to determine whether humans who experiment with the simultaneous use of cannabis and opioids report a more intense 'high' after such combinations or whether such an increase would affect the abuse potential of either drug. In most reported cases, the use of cannabis in opiate abusers takes place when they withdraw from opioids and is thought to represent an attempt to reduce withdrawal symptoms and craving for opiates (Taj *et al*, 1995). Nonetheless, the present results do suggest a mechanism by which marijuana consumption could potentially contribute to opioid dependence in poly-drug abusers.

In conclusion, opioid reinforcement was enhanced in this study by directly acting cannabinoid CB₁ receptor agonists, but not by compounds that indirectly increase the activity of endogenous cannabinoids by blocking their uptake or metabolism. These results clearly demonstrate that cannabinoid treatments can facilitate opioid reinforcement, but they do not provide support for the hypothesis that opioid reward is directly mediated by an opioid-induced release of endogenous cannabinoids. This would be in contrast with the reinforcing effects of cannabinoid drugs such as THC, whose psychotropic effects appear to be more directly mediated by a release of endogenous opioids in brain areas involved in reward processes, such as the VTA and nucleus accumbens (Solinas *et al*, 2004b). Finally, the findings that URB-597 and AM-404 did not enhance, and instead

reduced, the reinforcing efficacy of heroin indicates that these compounds would not pose a risk of increasing heroin drug-taking and drug-seeking in humans if they were used, as recently proposed (Piomelli, 2004), in the treatment of marijuana dependence and other aspects of drug abuse.

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