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Low dose combination of morphine and Δ^9 -tetrahydrocannabinol circumvents antinociceptive tolerance and apparent desensitization of receptors

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Abstract

Morphine and Δ^9 -tetrahydrocannabinol (THC) produce antinociception via mu opioid and cannabinoid CB₁ receptors, respectively, located in central nervous system (CNS) regions including periaqueductal gray and spinal cord. Chronic treatment with morphine or THC produces antinociceptive tolerance and cellular adaptations that include receptor desensitization. Previous studies have shown that administration of combined sub-analgesic doses of THC + morphine produced antinociception in the absence of tolerance. The present study assessed receptor-mediated G-protein activity in spinal cord and periaqueductal gray following chronic administration of THC, morphine or low-dose combination. Rats received morphine (escalating doses from 1–6 x 75 mg s.c. pellets or s.c. injection of 100 to 200 mg/kg twice daily), THC (4 mg/kg i.p. twice daily) or low-dose combination (0.75 mg/kg each morphine (s.c) and THC (i.p.) twice daily) for 6.5 days. Antinociception was measured in one cohort of rats using the paw pressure test, and a second cohort was assessed for agonist-stimulated [³⁵S]GTPγS binding. Chronic administration of morphine or THC produced antinociceptive tolerance to the respective drugs, whereas combination treatment did not produce tolerance. Administration of THC attenuated cannabinoid CB₁ receptor-stimulated G-protein activity in both periaqueductal gray and spinal cord, and administration of morphine decreased mu opioid receptor-stimulated [³⁵S]GTPγS binding in spinal cord or periaqueductal gray, depending on route of administration. In contrast, combination treatment did not alter cannabinoid CB₁ receptor- or mu opioid receptor-stimulated G-protein activity in either region. These results demonstrate that low-dose THC-morphine combination treatment produces antinociception in the absence of tolerance or attenuation of receptor function.

Keywords

Cannabinoid; Morphine; GTPγS; tolerance; paw-pressure test

1. Introduction

Both opioids and cannabinoids produce analgesia, hypothermia, sedation, hypotension, slowed intestinal motility, immunomodulation and motor depression (Holtzman et al., 1969; Bloom and Dewey, 1978). These effects are mediated by distinct opioid and cannabinoid receptors that are G-protein coupled and primarily activate G_i/G_o proteins (Childers, 1991; Howlett et

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al., 2002). Each receptor system includes multiple receptor subtypes; most clinically relevant analgesic opioids activate mainly mu opioid receptors and the CNS effects of cannabinoids are produced primarily by cannabinoid CB₁ receptors. Mu opioid and cannabinoid CB₁ receptors modulate a similar profile of effectors, including adenylyl cyclase, cAMP-dependent protein kinase (PKA), mitogen-activated protein kinase (MAPK), and voltage-dependent potassium and calcium channels (Childers, 1991;Howlett et al., 2002). Thus, the major intracellular effects of mu opioid or cannabinoid CB₁ receptor activation include decreased calcium and cAMP levels, which leads to inhibition of neurotransmitter release.

Opioids are considered highly effective analgesic agents, whereas clinical use of cannabinoids for treatment of pain remains controversial. A major limitation to the use of opioid analgesics is the development of tolerance and dependence with chronic use (Ellison, 1993). These adaptive changes have also been observed following chronic cannabinoid administration (Sim-Selley, 2003;Lichtman and Martin, 2005). At the cellular level, adaptation of G-protein-coupled receptors to chronic agonist administration occurs in two closely related steps: 1) uncoupling of receptor from G-protein activation (desensitization) and 2) internalization leading to receptor recycling or downregulation (Zhang et al., 1997). Chronic opioid administration produces region-specific desensitization of mu opioid receptors in the brain, with brainstem nuclei showing the greatest response (Noble and Cox, 1996;Sim et al., 1996b;Sim-Selley et al., 2000). Mu opioid receptor downregulation in brain occurs with certain chronic administration paradigms, but does not appear to be required for mu opioid receptor adaptation following chronic morphine treatment (reviewed in (Sim-Selley, 2005). Chronic administration of cannabinoids, such as THC, produces cannabinoid CB₁ receptor desensitization and downregulation (reviewed in (Sim-Selley, 2003). Although region-specific changes are found in cannabinoid CB₁ receptor adaptation, most CNS regions exhibit both responses following chronic cannabinoid treatment.

We have previously reported that simultaneous administration of sub-analgesic doses of morphine and THC in a 1:1 ratio (combination treatment) produces an analgesic effect equal to that of higher doses of either drug alone (Smith et al., 1998;Cichewicz et al., 1999) via a synergistic interaction (Cichewicz and McCarthy, 2003). We hypothesize that administration of the low-dose combination of THC and morphine circumvents the development of analgesic tolerance while producing similar analgesic efficacy as a higher dose of morphine or THC alone. The current studies investigated the mechanisms involved in prevention of tolerance to low-dose morphine-THC combination by examining receptor-mediated G-protein activity using agonist stimulated [³⁵S]GTPγS binding. The goal of this study was to characterize the effects of THC, morphine, or the combination of the two drugs on G-protein activation by mu opioid and cannabinoid CB₁ receptors. Studies were conducted in membranes prepared from periaqueductal gray or spinal cord because these regions have been implicated in opioid- and cannabinoid-mediated antinociception (Yaksh, 1981;Basbaum and Fields, 1984;Lichtman and Martin, 1991;Lichtman et al., 1996).

2. Methods

2.1 Animals

Male Sprague Dawley rats (Harlan Laboratories, Indianapolis IN) weighing approximately 325–350 grams were housed two per cage in animal quarters maintained at 22±2°C on a 12 hour light/dark cycle. Food and water were available *ad libitum*. The rats were brought to the test room prior to testing and were allowed to acclimate and recover from transport. 8–10 rats were used for each treatment group.

2.2 Drug administration Protocol

2.2.1 Morphine—Rats were rendered tolerant by subcutaneous (s.c.) implantation of morphine pellets (75 mg). Rats were anesthetized with isoflurane (Aerrane, Baxter Pharmaceutical Inc., Deerfield, IL) before shaving the hair around the base of the neck. Adequate anesthesia was noted by the absence of the righting-reflex and lack of response to the toe pinch. The skin was cleansed with 10% providone iodine (General Medical, Prichard, WV) and rinsed with alcohol before making a 1-cm horizontal incision at the base of the neck. The underlying s.c. space toward the dorsal flank was opened with a sterile glass rod. Maintenance of a stringent aseptic surgical field minimized any potential contamination of the pellet, incision, or subcutaneous space. On Day 1 one placebo pellet or one 75 mg morphine pellet was inserted into the space before closing the site with Clay Adams Brand, MikRon® AutoClip® 9 mm wound clips (Becton-Dickerson, Sparks, MD) and again applying iodine to the surface. The animals were allowed to recover in their home cages for 24 h. On day two, the rats were anesthetized and the incision was cleansed with 10% providone iodine and rinsed with alcohol. The incision was reopened and an additional 2 morphine or placebo pellets were implanted. The original pellet remained implanted. Thus, on Day 2 the rats had a total of 3 pellets implanted. After the addition of the pellets the surgical site was closed with the procedure outlined above. This procedure was repeated on day three, when 3 additional morphine or placebo pellets were implanted (making a total of 6 pellets per rat). Thus, each animal received implantation of a total of 6 pellets over the course of the study. Control rats received placebo pellets by the same surgical procedure described above (one pellet on Day 1, up to a total of 6 pellets on Day 3). Rats were tested for antinociception to a mechanical stimulus using the paw withdrawal test on days 1, 3, 5, and 7. In another cohort of rats, morphine was administered by s.c. injection of doses of 100 mg/kg twice daily (at 7:00 AM and 7:00 PM) for three days, followed by 150 mg/kg twice daily for two days, followed by 200 mg/kg twice daily for two days. These rats were tested for antinociception to a mechanical stimulus using the paw withdrawal test on days 1, 3 and 7.

2.2.2 THC—Tolerance to THC was achieved by administering 4 mg/kg of THC via intraperitoneal (i.p.) injection twice daily at 7:00 am and 7:00 pm for 6.5 days. Rats were tested 30 min after each injection on days 1, 3, 5 and 7 using the paw-withdrawal test. Control animals received twice daily i.p. injections of 1:1:18 (ethanol: emulphor: distilled water), which is the vehicle used to dissolve the THC.

2.2.3 Low-dose THC/Morphine Combination—Rats received 0.75 mg/kg of morphine sulfate s.c. and 0.75 mg/kg of THC i.p. administered twice daily at 12 hr intervals for 6.5 days. Control animals received an i.p. injection of 1:1:18 (ethanol: emulphor: distilled water) followed by s.c. injection of distilled water to correspond to the procedure used for drug-treated animals. Rats were tested 30 min after drug injection with the paw-withdrawal test on days 1, 3, 5 and 7. The low-dose combination of THC/morphine produced a full antinociceptive effect ($\geq 80\%$ Maximum Possible Effect (%MPE)).

2.3 Paw Withdrawal Test

The paw-pressure test consisted of gently holding the body of the rat while the hind-paw was exposed to increasing mechanical pressure. The Analgesy-Meter (Ugo-Basile, Varese, Italy) is designed to exert a force on the paw that increases at a constant rate, in a manner similar to the Randall and Selitto (Randall and Selitto, 1957) test of mechanical nociception. Force was applied to the hind paw that was placed on a small plinth under a cone-shaped pusher with a rounded tip. The operator depressed a pedal-switch to start the mechanism that exerted force. The force (measured in grams) at which the rat removed its paw was defined as the paw-pressure threshold. The baseline paw pressure was measured before injecting the vehicle or drug or pellet implantation. Antinociception was then quantified by calculating % Maximum

Possible Effect (% MPE) The mean response in grams was determined [$\pm$ standard error of the mean (S.E.M.)] 30 min post drug administration for each experiment with 8–10 rats per drug dose. The % MPE values for each group were compared statistically using repeated measures ANOVA followed by the Dunnett's t test for specific time points. The MPE is calculated as: $\%MPE = [(test-control) (500-control)^{-1}] \times 100$ with 500 g imposed for this set of experiments as the upper limit. The rats were tested twice daily every other day for seven days after the first pellet implantation or injection. Each daily average was recorded and then plotted in Sigma Plot®. In order to rule out the potential for repeated testing of the rats over the 7-day period leading to learning the withdrawal response, on the seventh day separate groups of rats that had been treated chronically with morphine, THC or the THC/morphine combination were tested for baseline response followed by acute challenge with either 40 mg/kg morphine sulfate for the morphine pellet implanted animals; 4 mg/kg THC for the THC-treated animals; or the combination of 0.75 mg/kg of THC and 0.75 mg/kg of morphine for the low dose combination-treated animals. Challenge doses were chosen based upon previous studies indicating that the challenge doses produced equi-efficacious effects in the paw-withdrawal test. The drug-challenged animals were tested 30 min after the injection in the paw-withdrawal test for antinociception. Tolerance was observed as a loss of antinociceptive effect of the challenge dose such that the average % MPE of the drug-treated groups did not significantly differ from the % MPE of rats treated chronically with vehicle. Statistical significance was determined using ANOVA followed by the Dunnett's t test.

Animal studies were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and adhere to the National Institute of Health Guide for the care and Use of Laboratory Animals (NIH Publications No. 80–23, 1996).

2.4 Tissue Preparation and Agonist-Stimulated [^{35} S]GTP γ S Binding Assay

After 6.5-days of treatment, a separate group of rats that received the chronic treatments described above were quickly sacrificed by decapitation. Rats from behavioral studies were not used for assay because challenge doses of drugs or behavioral manipulation could affect results. Whole spinal cords and the periaqueductal gray were removed and quickly frozen on dry ice and tissue was stored frozen at -80°C until assay. Agonist-stimulated [^{35} S]GTP γ S binding in membranes was performed by a modification of previously published methods (Sim et al., 1998). On the day of the assay, tissue was homogenized in ice-cold 50 mM Tris buffer (pH 7.4 at 30°C) with 3 mM MgCl_2 , 0.2 mM EGTA, and 100 mM NaCl (assay buffer) with a polytron homogenizer. The homogenate was centrifuged for 10 min at $50,000 \times g$ at 4°C . The pellet was resuspended in 10 ml of assay buffer. Protein levels were determined as described (Sim et al., 1998). Samples were not pooled, so that each N represents an individual rat. For each assay, tissue from a drug-treated rat and its appropriate control were run simultaneously.

Prior to assay, membranes (5–10 μg protein) were pre-incubated for 10 min at 30°C with adenosine deaminase (3 mU/ml) in assay buffer. Membranes (8–10 μg) were then incubated for 2 h at 30°C in assay buffer containing 0.1% bovine serum albumin (BSA), 30 μM GDP, 0.05 nM [^{35} S] GTP γ S and various concentrations of [D-Ala ^{2} ,N-MePhe ^{4} ,Gly ^{5} ol]enkephalin (DAMGO) or WIN55,212-2 (full agonists at μ opioid or cannabinoid CB $_1$ receptors, respectively) in a final volume of 0.5 ml. Basal binding was determined in the absence of agonist. Non-specific binding was measured in the presence of 20 μM unlabeled GTP γ S. The reaction was terminated by rapid vacuum filtration using a 96-well Brandel Harvester with Whatman GF/B glass fiber filters. The filters were rinsed three times with ice-cold 50mM Tris buffer, pH 7.2. After a 1 hour [^{35} S] extraction of the filters in 4 ml of Budget Solve® scintillation fluid, bound radioactivity was measured by liquid scintillation spectrophotometry at 95% efficiency for [^{35} S].

2.5 Data Analysis for Agonist-Stimulated [³⁵S]GTPγS Binding

Data are reported as a mean value ± S.E.M. of at least four separate experiments. Each individual sample was analyzed in triplicate. Net-stimulated [³⁵S]GTPγS binding is defined as stimulated binding minus basal binding. Percent control stimulation is defined as the net stimulated binding/net stimulated binding by a maximally effective concentration of agonist (20 μM DAMGO or 10 μM WIN55,212-2) in the corresponding vehicle-treated group × 100%. E_{max} and EC₅₀ values were calculated by non-linear regression analysis of concentration-effect curves with JMP (SAS® Institute, Cary, NC). Statistical significance of these data was determined with the non-paired two-tailed Student's *t*-test with Bonferroni adjustment for three comparisons with *p* 0.05 for significance.

2.6 Drugs and Chemicals

THC, DAMGO and morphine pellets were provided by the Drug Supply Program of the National Institute on Drug Abuse. [³⁵S]GTPγS was purchased from Perkin-Elmer Life Science (Boston, MA). GTPγS, GDP and WIN 55,212-2 were purchased from Calbiochem (San Diego, CA). Protein dye reagent was purchased from Bio-Rad Laboratories (Hercules, CA). All other reagent grade chemicals were purchased from Sigma Chemical Co. or Fisher Scientific.

3. Results

3.1 Paw Withdrawal

Rats were treated chronically with morphine, THC or a low-dose combination of the two drugs that produced 80% MPE, as described in Methods, and paw withdrawal thresholds were obtained on days 1, 3, 5, and 7. The results of paw withdrawal testing in high dose morphine treated rats are shown in Fig. 1A. The rats implanted with 75 mg morphine pellets exhibited significant antinociception compared to the placebo-pellet implanted rats on day 1 (*p* < 0.001, ANOVA with post-hoc Newman-Keuls test). By day 3 of testing, the morphine-pellet implanted rats had a significant decrease in antinociception compared to day 1 (*p* < 0.01, ANOVA with post-hoc Dunnett's test). At days 5 and 7 there was no significant difference between the morphine-treated and placebo-treated groups (ANOVA with post-hoc Newman-Keuls test), demonstrating that tolerance had developed in rats that received morphine pellets. A separate group of rats received morphine injections to match the route of administration of THC and low-dose drugs, and to confirm that these results were not due to morphine administration via pellet implantations (Figure 1D). Similar results were obtained in rats that received injections as described for morphine pellet implanted rats. Injection of 100 mg/kg morphine produced 100% antinociception on day 1 (*p* < 0.01, ANOVA with post-hoc Newman-Keuls test). In contrast, doses of 100 and 200 mg/kg morphine produced significantly less antinociception on day 3 and 7, respectively, than that obtained with the 100 mg/kg dose of morphine on day 1 (*p* < 0.01, ANOVA with post-hoc Dunnett's test). Moreover, morphine-induced antinociception in this treatment paradigm did not differ from that of vehicle on days 3–7 (ANOVA with post-hoc Newman-Keuls test). Thus, tolerance to morphine was observed regardless of the method of administration.

Rats treated twice daily with 4 mg/kg of THC showed a similar development of tolerance (Fig. 1B). THC-treated rats exhibited antinociception compared to vehicle-treated rats in the paw withdrawal test on day 1 (*p* < 0.001, ANOVA with post-hoc Newman-Keuls test). Antinociception in THC-treated rats on days 3, 5, and 7 was significantly decreased compared to the antinociception observed on day 1 (*p* < 0.01, ANOVA with post-hoc Dunnett's test). By day 5, no significant difference in antinociception was observed between the vehicle- and THC-treated rats (ANOVA with post-hoc Newman-Keuls test). Thus, THC-treated rats exhibited antinociceptive tolerance.

In contrast to the results obtained in the high dose treatment groups, rats treated with the low dose combination of morphine (0.75mg/kg, s.c., twice daily) and THC (0.75mg/kg, i.p., twice daily) showed no change in their response in the paw withdrawal test during the course of the treatment period (Fig. 1C). The antinociceptive efficacy of this low dose combination was similar to that of the high dose of either morphine or THC alone. Paw withdrawal thresholds of the morphine + THC combination-treated rats were significantly greater than vehicle-treated rats throughout the course of treatment (ANOVA with post-hoc Newman-Keuls test). Moreover, the magnitude of antinociception produced by the low dose combination throughout the time course of treatment was statistically indistinguishable from the initial effect observed on day 1 (ANOVA with post-hoc Dunnett's test). Thus, tolerance did not develop to the low dose combination of morphine and THC in the paw-withdrawal test.

Additional treatment groups were tested with 0.75 mg/kg of morphine or THC alone, and no significant antinociception was observed over the 7-day period (ANOVA with post-hoc Newman-Keuls test). The low dose of THC in combination with the vehicle for morphine (distilled water) produced 6 ± 8 , 7 ± 2.6 and $6.1 \pm 1.7\%$ MPE on days 1, 3 and 7, respectively. Low dose morphine in combination with the vehicle for THC (1:1:18; emulphor:ethanol:saline) produced 18.2 ± 4.9 , 11.4 ± 2.9 and $7.3 \pm 2.4\%$ MPE on days 1, 3 and 7 respectively. These values did not differ significantly from those obtained with the dual vehicle injection of distilled water and 1:1:18; emulphor:ethanol:saline, which were: 3.9 ± 2.7 , 14.6 ± 7 , $14.6 \pm 1.6\%$ MPE on days 1, 3 and 7, respectively.

The results described above were generated by repeated testing of rats over the 7 day period. In order to verify that loss of paw withdrawal represented tolerance rather than a learned behavior, separate groups of animals were treated chronically with morphine, THC, or the low-dose combination of THC/morphine and tested only once at each time point (3 days, 5 days, or 7 days). The results of these studies at single time points were the same as those obtained with repeated testing (data not shown), confirming that tolerance developed to high doses of morphine or THC alone, but not the low-dose THC/morphine combination.

3.2 DAMGO-stimulated [^{35} S]GTP γ S Binding

Mu opioid receptor-stimulated [^{35} S]GTP γ S binding was examined in control or chronic drug-treated rats after 7 days to determine whether the different treatments produced a change in mu opioid receptor-mediated G-protein activation. Concentration-effect curves were generated using the mu opioid receptor-selective full agonist DAMGO in membranes prepared from spinal cords of rats implanted with morphine pellets, injected with high dose morphine, injected with high dose THC, injected with the low dose morphine/THC combination or their respective control groups. Morphine pellet-implanted rats showed a 22% decrease in the $E_{\max}$ value of DAMGO-stimulated [^{35} S]GTP γ S binding compared to placebo pellet-implanted rats, with no significant difference in EC_{50} values (Fig. 2A, Table 1). In contrast, there was no difference in DAMGO $E_{\max}$ or EC_{50} values between vehicle-treated and THC- or low dose THC + morphine-treated rats (Fig. 2A, Table 1). Interestingly, there were also no differences between the $E_{\max}$ or EC_{50} values of morphine-injected compared to vehicle-injected rats in spinal cord (Table 1). Basal [^{35} S]GTP γ S binding also did not differ between any of the drug-treated groups of rats and their respective vehicle controls (not shown).

DAMGO-stimulated [^{35} S]GTP γ S binding was also examined in membranes prepared from periaqueductal gray of morphine-pelleted, morphine-injected, THC-injected and low dose THC + morphine combination-injected rats. In contrast to spinal cord, neither the $E_{\max}$ nor EC_{50} value of DAMGO-stimulated [^{35} S]GTP γ S binding was significantly altered in morphine-pelleted compared to placebo-pelleted rats (Fig. 2B, Table 1). However, the $E_{\max}$ value of DAMGO-stimulated [^{35} S]GTP γ S binding in periaqueductal gray was decreased in morphine-injected compared vehicle-injected rats, with no difference in EC_{50} value (Table 1). Similarly

to the spinal cord, no significant differences were seen in DAMGO-stimulated [^{35} S]GTP γ S binding in periaqueductal gray membranes prepared from vehicle-treated versus THC- or low dose THC + morphine combination-treated animals (Fig. 2B, Table 1). Basal [^{35} S]GTP γ S binding also did not differ between any of the drug-treated groups and their respective vehicle controls (not shown). These results indicate that antinociceptive tolerance to morphine in the paw pressure test was associated with attenuation of mu opioid receptor-mediated G-protein activation in the spinal cord but not periaqueductal gray of morphine-pelleted rats, whereas in rats injected with morphine, mu opioid receptor-mediated G-protein activity was attenuated in PAG only. Moreover, treatment with either 4 mg/kg THC alone or the low-dose THC + morphine combination did not alter mu opioid receptor-mediated G-protein activation in either region.

3.3 WIN 55,212-2-stimulated [^{35} S]GTP γ S Binding

To examine cannabinoid CB $_1$ receptor-stimulated G-protein activity in rats that were treated chronically with morphine via pellet implantation, THC, or a low dose combination of morphine + THC for 6.5 days, WIN 55,212-2-stimulated [^{35} S]GTP γ S binding was performed (Fig. 3). Results showed that there was a 50% decrease in the $E_{\max}$ value of WIN 55,212-2-stimulated [^{35} S]GTP γ S binding in spinal cord membranes prepared from THC- versus vehicle-treated rats (Fig. 3A, Table 2). The EC_{50} values of WIN 55,212-2-stimulated [^{35} S]GTP γ S binding appeared to increase by >4-fold in THC-treated versus vehicle-treated rat spinal cord, but this apparent difference was not statistically significant. In contrast, $E_{\max}$ and EC_{50} values of WIN 55,212-2 did not differ between vehicle-treated and morphine-treated or low dose THC + morphine combination-treated rats (Fig. 3A, Table 2). Basal binding [^{35} S]GTP γ S also did not differ between tissue from vehicle-treated versus any of the drug-treated groups of rats (not shown).

WIN 55,212-2-stimulated [^{35} S]GTP γ S binding in periaqueductal gray membranes prepared from rats in the three treatment groups yielded similar results to those observed in spinal cord. The $E_{\max}$ value of WIN 55,212-2-stimulated [^{35} S]GTP γ S binding was decreased by 27% in chronic THC-treated compared to vehicle-treated rats, without a significant difference in EC_{50} values (Fig. 3A, Table 2). However, no significant differences were observed in the $E_{\max}$ or EC_{50} values of WIN 55,212-2 between vehicle-treated and morphine-pellet implanted or low dose combination THC + morphine-treated rats (Fig. 3B, Table 2). As in spinal cord, none of the drug treatments significantly altered basal [^{35} S]GTP γ S binding in periaqueductal gray (not shown). These results indicate that tolerance to the antinociceptive effects of THC in the paw pressure test was associated with attenuation of cannabinoid CB $_1$ receptor-mediated G-protein activation in both spinal cord and periaqueductal gray. In contrast, no alteration in cannabinoid CB $_1$ receptor-mediated G-protein activation was observed in either region of rats treated with morphine alone or with the low dose combination of THC + morphine.

Discussion

Morphine and THC are effective in producing analgesia, but can produce dose-related side effects. Moreover, long-term administration of therapeutic doses of either morphine or THC results in the development of tolerance and physical dependence, thus reducing analgesic potency and necessitating the administration of higher drug doses which are associated with increased side-effects. Therefore, low dose combination therapy might offer a safer, more effective alternative than either drug alone by decreasing the development of tolerance to the drug-induced analgesia. Combination treatment involves the simultaneous administration of both drugs in a 1:1 ratio of sub-efficacious doses to produce an analgesic effect equal to that of either drug alone. This concept is based on our previous studies showing that a low dose of THC greatly enhances the antinociceptive effect of morphine (Smith et al., 1998; Cichewicz et

al., 1999) in a synergistic manner (Cichewicz and McCarthy, 2003). This enhancement of opioid antinociception by cannabinoid agonists has been demonstrated using not only morphine and THC, but also other opioid and cannabinoid agonists (for review see (Cichewicz, 2004). Moreover, this enhancement has been reported via central, parenteral, oral and transdermal routes of administration (Cichewicz, 2004;Cichewicz et al., 2005). Most previous tests of cannabinoid-enhanced opioid antinociception focused on thermal stimulation of the tail, whereas the present study is the first, to our knowledge, to examine the effect of the cannabinoid/opioid combination on nociceptive response to mechanical stimulation of the paw.

The present results showed that both morphine and THC are fully efficacious in the paw pressure test, whether alone or combined in a synergistic low dose combination. It is important to note that although the doses of THC, morphine and the THC + morphine combination administered in the present study were equally effective in producing antinociception, much higher doses of morphine or THC alone were required to produce $\geq 80\%$ antinociceptive effect compared to the doses administered during combination treatment. We further demonstrated that administration of the low dose combination of THC and morphine circumvents morphine tolerance in the paw pressure test for antinociception while maintaining similar efficacy as a higher dose of morphine or THC alone. In contrast, antinociceptive doses of morphine and THC alone produced profound tolerance after repeated administration over the one-week time course.

Compensatory adaptations of mu opioid and cannabinoid CB₁ receptors occur in response to persistent receptor occupation by an agonist. The process of GPCR adaptation has previously been reviewed (Zhang et al., 1997). Cellular adaptation begins with activation of the G-protein, which leads to dissociation of the α and $\beta\gamma$ subunits. The $\beta\gamma$ subunit recruits certain G-protein coupled receptor kinase (GRK) subtypes to the membrane, which in turn phosphorylate the agonist-bound receptor. The phosphorylated receptor becomes a substrate for β -arrestin association, which results in uncoupling of the receptor from G-protein activation. β -arrestin also promotes receptor internalization, leading to recycling to the plasma membrane or degradation of the receptor. Both mu opioid and cannabinoid CB₁ receptors exhibit this adaptive mechanism in cell culture models, resulting in receptor desensitization and internalization (Kovoor et al., 1997;Zhang et al., 1998;Hsieh et al., 1999;Jin et al., 1999).

Adaptation of mu opioid and cannabinoid CB₁ receptors in brain can differ somewhat from cell culture models. Moreover, regional differences have been identified in the magnitude and temporal components of desensitization and downregulation. Autoradiographic analysis of cannabinoid CB₁ receptor-mediated G-protein activity using agonist stimulated [³⁵S]GTP γ S binding has shown that desensitization and downregulation occur in most cannabinoid CB₁ receptor-containing regions of brains from THC-treated animals (reviewed by Sim-Selley, 2003). These adaptive responses are large in magnitude and regionally widespread, and include the periaqueductal gray and spinal cord. Chronic morphine treatment produces more profound regional differences in mu opioid receptor adaptation. Mu opioid receptor desensitization is found in most brainstem regions and spinal cord following most chronic administration paradigms, whereas forebrain regions such as the striatum appear resistant (reviewed in Sim-Selley, 2005). Downregulation does not appear to be a fundamental response to chronic morphine administration in vivo, although certain chronic opioid administration paradigms result in mu opioid receptor downregulation (reviewed in Sim-Selley, 2005). Thus, the spinal cord and periaqueductal gray seem to be two regions that are susceptible to agonist-induced desensitization of both mu opioid and cannabinoid CB₁ receptors.

The current study addressed the hypothesis proposed by Cichewicz and Welch (Cichewicz and Welch, 2003) that tolerance is avoided in low dose combination treatment because cellular adaptations do not occur, by directly measuring receptor-mediated G-protein activation in two

CNS regions that are known to be involved in both opioid- and cannabinoid-mediated antinociception: spinal cord and periaqueductal gray. In the present study, attenuation of cannabinoid CB₁ receptor-mediated G-protein activation was found in the spinal cord and periaqueductal gray of chronic THC-treated animals, while chronic morphine treatment attenuated mu opioid receptor-mediated G-protein activation in the spinal cord or PAG, depending on the administration paradigm. The reason for this regional difference in mu opioid receptor adaptation based on paradigm of administration is unclear. However, previous studies in both rats and mice have found that the dose and administration pattern of morphine can affect the magnitude and regional distribution of attenuation of mu opioid receptor-mediated G-protein activation (Sim et al., 1996b;Kruzich et al., 2003;Sim-Selley et al., 2007). There could be multiple reasons for this regional disparity in sensitivity of mu opioid receptors to the effects of different chronic morphine administration paradigms. Potential mechanisms include differential distribution of mu opioid receptor isoforms or oligomers, and differential co-localization of mu opioid receptors with specific types of various signaling or regulatory proteins, including GRK, β -arrestin, regulators of G-protein signaling (RGS) or G-protein subunits (for review see Sim-Selley, 2005). Nonetheless, the low dose combination of morphine + THC did not alter receptor-mediated G-protein activation in either region. Receptor binding was not examined in the present study; therefore it is not known whether receptor downregulation was produced by these chronic administration paradigms. However, the lack of apparent desensitization observed following chronic THC/morphine administration indicates that mu opioid and cannabinoid CB₁ receptor signaling was unchanged after this treatment.

The results of the present study are similar to other studies that have shown apparent mu opioid receptor desensitization in the spinal cord or PAG following chronic administration of morphine (Sim et al., 1996b;Maher et al., 2001;Sim-Selley et al., 2007). Similarly, chronic THC administration has been shown to produce apparent CB₁ receptor desensitization in PAG (Sim et al., 1996a;Sim-Selley and Martin, 2002). This is to our knowledge the first demonstration that cannabinoid-mediated G-protein activation is attenuated in spinal cord by chronic THC treatment. These results further suggest that antinociceptive tolerance to morphine or THC could be associated with desensitization of receptors in PAG and/or spinal cord. The current study used membrane homogenates prepared from whole spinal cord and periaqueductal gray, but antinociceptive interactions between opioids and cannabinoids could occur via activity in specific anatomical loci where both receptors are co-localized. Co-localization of mu opioid and cannabinoid CB₁ receptors has been identified in lamina II interneurons of the dorsal horn of the spinal cord (Salio et al., 2002). To our knowledge, no studies have yet demonstrated co-localization of mu opioid and CB₁ receptors in specific subnuclei of the periaqueductal gray. It will be of interest in future studies to further define the anatomical substrates underlying the synergistic effects of cannabinoid and opioid agonists in producing antinociception.

The mechanism by which THC and morphine in combination produce antinociception without tolerance is not known, but the synergistic antinociceptive response to this drug combination appears to require cannabinoid CB₁ receptor-mediated release of endogenous opioid peptides (Cichewicz, 2004). Moreover, the data support the hypothesis that tolerance does not develop to the low dose combination of morphine and THC because cellular adaptations leading to receptor adaptations, including attenuation of receptor-mediated G-protein activation, do not occur. It has been suggested that all three opioid receptor types might play roles in producing antinociceptive synergy (Sutters et al., 1990;Cichewicz, 2004). Cichewicz and Welch (2003) proposed that THC and morphine administered in a low dose combination prevents adaptation in mu, delta and kappa opioid receptors, thus maintaining the activity of the endogenous opioid system. Thus, lack of tolerance development to the combination treatment might be associated with the low doses of drugs used. The doses used in the combination treatment might produce

insufficient occupancy and stimulation of receptors to produce desensitization, downregulation or downstream adaptations in signaling pathways that could lead to tolerance development.

The antinociceptive synergism between opioids and cannabinoids in animal models suggests the possibility of clinical utility in humans. Two recent studies examined the effectiveness of morphine + THC combination in acute pain in human volunteers (Naef et al., 2003; Roberts et al., 2006). While both showed slight enhancement of certain aspects of opioid analgesia by THC, the overall results indicated limited antinociceptive effect of the combination. However, both studies focused on a limited dose range. Furthermore, no studies to date have examined the effect of longer duration treatment with opioid/cannabinoid combinations in humans, and our results suggest that prevention of tolerance could be a key advantage of the combination treatment. Moreover, we recently showed that THC can prolong the duration of opioid action when administered subsequent to the opioid (Williams et al., 2006), but this aspect of the combination has not been examined in humans. Finally, it has consistently been shown that THC enhances antinociception of codeine to a greater extent than that of morphine (Cichewicz, 2004), but this combination has not been tested clinically.

In summary, chronic treatment with the low dose THC + morphine combination does not produce tolerance in the paw pressure test, nor does it produce attenuation of mu opioid or cannabinoid CB₁ receptor-mediated G-protein activation in the periaqueductal gray or spinal cord of these animals. The lack of tolerance development is consistent with previous observations (Cichewicz and Welch, 2003). From these results we conclude that chronic administration of an equi-efficacious, low dose combination of morphine and THC does not produce long-term apparent desensitization of either receptor. These results suggest that tolerance to antinociceptive effects of opioids or cannabinoids is not observed when the doses used do not produce receptor adaptation, but do not necessarily indicate that receptor desensitization or downregulation are solely responsible for antinociceptive tolerance. The interaction of opioid and cannabinoid systems to produce antinociception in the absence of tolerance provides evidence that it might be possible to enhance the analgesic properties of these drugs clinically and minimize the side-effects associated with higher doses of either drug alone. However, further research in human subjects is needed to determine whether there is clinical utility for this drug combination treatment.

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Fig 1.

The effects of chronic morphine pellet implantation (6 x 75 mg pellets)(A), chronic THC (4 mg/kg i.p, twice daily) (B), low dose combination (0.75 mg/kg each morphine (s.c) and THC (i.p.) twice daily)(C) or morphine injection (100, 150 and 200 mg/kg (s.c.) twice daily) (D) in the paw withdrawal test over a 7-day period. Data are mean percent MPE $\pm$ S.E., with each point representing 8–10 rats in the paw pressure test. *, **,***: $p < 0.05$, 0.01 or 0.001, respectively, different from vehicle treated mice determined by ANOVA with post-hoc Newman-Keuls test. †,††: $p < 0.05$, 0.01, respectively, different from day 1 within the same treatment group determined by repeated measures ANOVA with post-hoc Dunnett's test.

Fig 2.

Effect of chronic morphine pellet implantation (6 x 75 mg pellets) on DAMGO-stimulated [^{35} S]GTP γ S binding. Membranes from spinal cord (A) or periaqueductal gray (B) of vehicle or morphine pellet-implanted rats were incubated with 0.05 nM [^{35} S]GTP γ S, 30 μ M GDP and the indicated concentrations of DAMGO. Data are mean percent control stimulation $\pm$ S.E from 4–7 rats per treatment group.

Fig 3.

Effect of chronic THC treatment (4 mg/kg i.p, twice daily) on WIN55,212-2-stimulated [^{35} S] GTP γ S binding. Membranes from spinal cord (A) or periaqueductal gray (B) of vehicle or THC treated rats were incubated with 0.05 nM [^{35} S]GTP γ S, 30 μ M GDP and the indicated concentrations of WIN55,212-2. Data are mean percent control stimulation $\pm$ S.E from 4–7 rats per treatment group.

Table 1

Effect of morphine, THC or low-dose combination treatment on $E_{\max}$ and EC_{50} values of mu opioid receptor-stimulated [^{35} S]GTP γ S binding

Region Spinal Cord	Vehicle Control		Drug Treated	
	$E_{\max}$ (% Con)	EC_{50} (nM)	$E_{\max}$ (% Con)	EC_{50} (nM)
Morphine (pellet)	100 $\pm$ 5.5	214 $\pm$ 11	78 $\pm$ 2.2 ^a	143 $\pm$ 33
Morphine (injection)	100 $\pm$ 2.0	189 $\pm$ 72	97 $\pm$ 6.4	201 $\pm$ 61
THC	100 $\pm$ 16	97 $\pm$ 49	94 $\pm$ 5.1	61 $\pm$ 26
Combo	100 $\pm$ 8.7	91 $\pm$ 47	88 $\pm$ 10	183 $\pm$ 76
Periaqueductal Gray	$E_{\max}$ (% Con)	EC_{50} (nM)	$E_{\max}$ (% Con)	EC_{50} (nM)
Morphine	100 $\pm$ 6.9	113 $\pm$ 22	87 $\pm$ 9.9	149 $\pm$ 33
Morphine (injection)	100 $\pm$ 2.8	167 $\pm$ 47	81 $\pm$ 3.1 ^a	137 $\pm$ 33
THC	100 $\pm$ 3.4	53 $\pm$ 10	98 $\pm$ 2.5	76 $\pm$ 15
Combo	100 $\pm$ 11	113 $\pm$ 34	96 $\pm$ 8.0	128 $\pm$ 49

Membranes were incubated with 0.1 nM [^{35} S]GTP γ S, 30 μ M GDP and varying concentrations of DAMGO, as described in Methods. Data are $E_{\max}$ and EC_{50} values derived from the concentration-effect curves shown in Fig. 1.

^aSignificant from vehicle control by Bonferroni-adjusted Student's *t*-test.

Table 2

Effect of morphine, THC or low-dose combination treatment on $E_{\max}$ and EC_{50} values of cannabinoid CB_1 receptor-stimulated [^{35}S]GTP γ S binding

Region Spinal Cord	Vehicle Control		Drug Treated	
	$E_{\max}$ (% Con)	EC_{50} (nM)	$E_{\max}$ (% Con)	EC_{50} (nM)
Morphine	100 ± 18	93 ± 14	106 ± 9.4	51 ± 20
THC	100 ± 11	40 ± 4.8	50 ± 6.4 ^a	190 ± 45
Combo	100 ± 21	57 ± 9.7	89 ± 10	50 ± 17
Periaqueductal Gray	$E_{\max}$ (% Con)	EC_{50} (nM)	$E_{\max}$ (% Con)	EC_{50} (nM)
Morphine	100 ± 8.7	219 ± 42	103 ± 14	273 ± 34
THC	100 ± 4.8	148 ± 41	73 ± 6.6 ^a	295 ± 95
Combo	100 ± 16	215 ± 67	91 ± 7.5	157 ± 31

Membranes were incubated with 0.1 nM [^{35}S]GTP γ S, 30 μ M GDP and varying concentrations of WIN55,1212-2, as described in Methods. Data are $E_{\max}$ and EC_{50} values derived from the concentration-effect curves shown Fig. 2.

^aSignificantly different from vehicle control by Bonferoni-adjusted Student's *t*-test.