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Decreased basal endogenous opioid levels in diabetic rodents: Effects on morphine and delta-9-tetrahydrocannabinoid-induced antinociception

Jovan Williams, Victoria L. Haller *, David L. Stevens, Sandra P. Welch

Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298-0524, United States

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Abstract

We have previously demonstrated synergy between morphine and Δ^9-tetrahydrocannabinol (Δ^9-THC) in the expression of antinociception in acute pain models and in arthritic models of chronic pain. Our data has been extended to include acute pain in both diabetic mice and rats. In diabetic mice, Δ^9-THC p.o. was more active in the tail-flick test in the diabetic mouse than in the non-diabetic mouse. Morphine (s.c.) was less potent in diabetic than in non-diabetic mice [6.1 (5.1–7.2) versus 3.2 (2.4–4.1) mg/kg, respectively], an effect previously extensively documented in pre-clinical and clinical testing. In addition, the combination of Δ^9-THC with morphine produced a greater-than-additive relief of acute pain in mice. In the rat, the induction of the diabetic state decreased the antinociceptive effect of morphine, an effect temporally related to a decreased release of specific endogenous opioids. Conversely, Δ^9-THC retained the ability to release endogenous opioids in diabetic rats and maintained significant antinociception. Extrapolation of such studies to the clinical setting may indicate the potential for use of Δ^9-THC-like drugs in the treatment of diabetic neuropathic pain, alone or in combination with very low doses of opioids.
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Keywords: Opioid; Cannabinoid; Diabetes

1. Introduction

It is estimated that 2 to 3 million patients with diabetes have neuropathy as a result of their disease, and that 10% of diabetic patients are affected by pain associated with diabetic neuropathy (Harati, 1996). Studies have shown that both opioids and cannabinoids are effective antinociceptive drugs in normal animals without pathological pain. However, these antinociceptive effects differ in pathological pain states, and opioid antinociception is reduced in painful peripheral neuropathy such as that associated with diabetes, whereas cannabinoid antinociception is not changed (Mao et al., 2000). Interactions between Δ^9-tetrahydrocannabinol (Δ^9-THC) and the endogenous opioid/dynorphin A system are proposed to contribute to Δ^9-THC-induced antinociception in acute pain models such as the tail-flick and hot-plate tests (Mason et al., 1999; Welch and Eads, 1999; Cox and Welch, 2004). Δ^9-THC stimulates the release of the endogenous κ-opioid agonist, dynorphin, which contributes to the antinociceptive effects of Δ^9-THC (Pugh et al., 1996). Δ^9-THC analgesia is significantly reduced in dynorphin-deficient mice (Zimmer et al., 2001). Recently it has been shown that Δ^9-THC differentially modulates endogenous opioids in normal versus arthritic rats depending on basal dynorphin levels (Cox and Welch, 2004), suggesting that the cannabinoid receptor may serve as a homeostatic modulator of the tonic release of opioids in the spinal nociceptive pathways. That is, in the arthritic rat, basal dynorphin A levels and release are substantially higher in the spinal cord compared to the levels in the normal, non-arthritic rat. Δ^9-THC administration significantly reduces the high dynorphin release in the arthritic rats, an effect correlated with the antinociceptive effects of Δ^9-THC (Cox and Welch, 2004). Conversely, Δ^9-THC administration to non-arthritic rats increases dynorphin A release, an effect temporally correlated to the antinociceptive effects of Δ^9-THC. Thus, the effects of Δ^9-THC on dynorphin release differ based upon endogenous opioid tone. Such results led to the hypothesis that Δ^9-THC might have a similar "normalizing" effect in a disease state in which we observe low levels of selective endogenous

* Corresponding author. Department of Pharmacology and Toxicology, P.O. Box 980524, Richmond, VA 23298-0524, United States. Tel.: +1 804 828 8446; fax: +1 804 828 1532.
E-mail address: vhaller@vcu.edu (V.L. Haller).

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opioids with streptozotocin-induced diabetes in the rat. In addition, Hohmann and Herkenham (1998) have shown dissociation between cannabinoid and opioid receptor sites in the spinal cord neurons. They hypothesize that neuropathy is associated with a decrease in presynaptic opioid receptors in spinal afferents. They show that few cannabinoid receptors are located at similar sites. Thus, cannabinoids are hypothesized to maintain activity in neuropathic pain treatment, while opioid treatment has reduced efficacy. Based upon their work, we hypothesize that it is possible that the combination of Δ^9-THC and opioids may enhance and restore the efficacy of opioids. The two main goals of this research were to determine whether the greater-than-additive effect of Δ^9-THC in combination with morphine as seen in naïve mice, rats, and arthritic rats (Cox et al., 2007a) shows similar actions in diabetic mice and rats and to determine which endogenous opioids are involved in the elicitation of the enhanced antinociceptive effect. In addition, although we did not examine neuropathic pain, based upon the studies of Hohmann and Herkenham (1998) we hypothesized that the potency of Δ^9-THC in diabetic mice and rats would equal or surpass that in non-diabetic mice or rats in contrast to morphine that would have reduced potency in diabetics. We further hypothesized that a potential mechanism underlying the loss of potency of morphine in diabetic rats would be the loss of basal endogenous opioid tone and loss of endogenous opioid release by morphine in diabetic rats. Conversely, if Δ^9-THC retained antinociceptive potency in the diabetic animal, we hypothesized that Δ^9-THC would retain the ability to release endogenous opioids previously shown responsible for its antinociceptive effects, namely dynorphin and leucine enkephalin. Our study represents the first direct quantification of the basal decrease in specific endogenous opioids in cerebrospinal fluid due to the induction of diabetes. In addition, although many theories as to the decrease in the potency of morphine in diabetic animals and humans have emerged, our work is the first proposal that morphine fails to release endogenous opioids in the diabetic, an effect that could underlie its lack of potency. In addition, our study addresses the proposal that activation of the cannabinoid receptor with Δ^9-THC results in a "normalization" of endogenous opioid levels, resulting in an antinociceptive effect of Δ^9-THC in the diabetic rodent. We hypothesize that such results may indicate the clinical utility of cannabinoid-like drugs (or drugs that increase endocannabinoids) as either a monotherapy or adjunct to opioids in the treatment of pain in diabetics.

2. Methods

2.1. Animals

Male ICR mice (Harlan Laboratories, Indianapolis, IN) weighing 25–30 g and male Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 350–375 g were housed in an animal care facility maintained at 22±2°C on 12-h light/dark cycle. Food and water were available ad libitum. The animals were transported to the testing laboratory 24 h prior to the test day to allow acclimation and recovery from transport and handling. All experiments were conducted in accordance with the regulations established by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

2.2. Drugs

Morphine sulfate and Δ^9-THC were obtained from the National Institute on Drug Abuse (Rockville, MD), and streptozotocin was obtained from Calbiochem (La Jolla, CA). Morphine and streptozotocin were dissolved in distilled water (vehicle), and Δ^9-THC was prepared in 1:1:18 (emulphor:ethanol:saline).

2.3. Induction of diabetes

Prior to testing day, animals were weighed and injected with streptozotocin, a diabetogenic agent, 170 mg/kg, i.p. for the mice and 75 mg/kg, i.p. for the rats in a single dose for each species. Two weeks were allowed for destruction of pancreatic beta cells and development of Type 1 diabetes mellitus. Blood glucose levels were measured using capillary retro-orbital sinus blood collection and animals exhibiting blood glucose levels >400 mg/dl (average 590 mg/dl) were used in the study.

2.4. Antinociception in mice and rats

Mice were employed in initial antinociceptive studies based upon our previous studies of the synergy of morphine and Δ^9-THC (Cichwicz et al., 1999; Cichewicz and McCarthy, 2003). For studies in mice, morphine was administered s.c. and Δ^9-THC was administered p.o. (both 0.1 cc/gm body weight of the mouse) in groups of six animals. The vehicle for morphine was distilled water and for Δ^9-THC was 1:1:18 (emulphor:ethanol: saline). The rats tested were part of a larger study of various chronic pain conditions of which data on arthritic rats has been previously published (Cox and Welch, 2004; Cox et al., 2007a). In those studies, morphine and Δ^9-THC were administered i.p. with the vehicles as described for the mice. For both mice and rats, baseline tail-flick latencies were determined prior to drug administration using the tail-flick latency test for antinociception as designed by D'Amour and Smith (1941). Baseline values were between 2 and 4 s. During testing the cutoff time of 10 s was employed to prevent damage to the tail. Antinociception was quantified using the percent maximal effect (%MPE) calculated as developed by Harris and Pierson (1964):%MPE= [(test−baseline)/(10−baseline)]×100. Using the %MPE for each animal, the mean effect and standard error of the mean (S.E.M.) were recorded for each of the respective groups. Effective dose 50% (ED_{50}) values and 95% confidence limits (CL) were calculated using a program modified from Bliss (1967). ED_{50} values were determined to differ significantly when the 95% confidence limits did not overlap.

2.5. Rat spinal perfusion

Rats were employed in endogenous opioid studies for retrieval of adequate quantities of cerebrospinal fluid unattainable in the smaller mice species. Rats were anesthetized via an i.

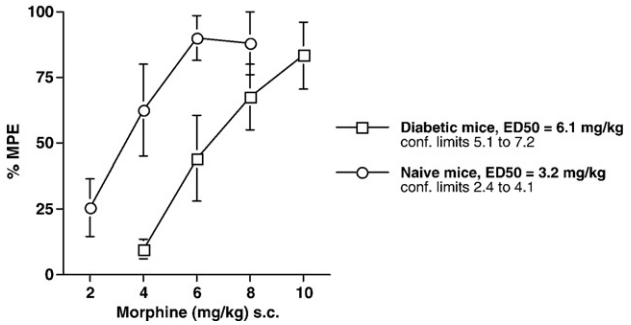

Fig. 1. The antinociceptive effect of morphine, s.c. in diabetic and non-diabetic (naïve) mice in the mouse tail-flick test. Mice were injected with morphine in doses ranging from 2–10 mg/kg, s.c. and tested in the tail-flick test 20 min post-administration to evaluate the antinociceptive effect of morphine. Morphine, s.c. was found to have decreased antinociceptive potency in diabetic mice as compared to naïve mice at all doses resulting in a rightward shift in its DRC and an increase in its ED_{50} from 3.2 mg/kg [2.4–4.1] in naïve mice to 6.1 mg/kg [5.1–7.2] in diabetic mice. %MPE = percent maximal effect; ED_{50} = effective dose 50%; conf. limits = 95% confidence limits; THC = Δ^9-THC 20 mg/kg, p.o.; vehicle (1:1:18) = ethanol:emulphor:saline; BG = blood glucose; V = vehicle; M = morphine; T = Δ^9-THC; D = diabetic.

p. injection of sodium barbital (375 mg/kg) and a separate i.p. injection of 2 mg/kg atropine methyl nitrate. The anesthetized rats were placed in stereotaxis and an incision made on the atlanto–occipito membrane to expose the cisterna magna. A catheter of PE-10 polyethylene tubing was inserted through the exposed cisternal cavity, caudally, into the subarachnoid space of the spinal cord. The catheter contained an artificial cerebrospinal fluid composed of 125 mM Na^+; 2.6 mM K^+; 0.9 mM Mg^{2+}; 1.3 mM Ca^{2+}; 122.7 mM Cl^-; 21.0 mM HOC^-; 2.4 mM HOP^{2-}; 0.5 mg/ml bovine serum albumen; bacitracin (30 mg/ml); and 0.01% Triton X and effervesced with 95% O_2 and 5% CO_2. Positioned as such, the catheter extends caudally 8.5 cm passing through the thoracolumbar region to an area just above the sacral enlargement. Following catheter implantation, animals were allowed to acclimate approximately 30 min on a heating pad. Following acclimation, baseline tail-flick latency was assessed. Acceptable latency measurements were 1.5–4 s to avoid use of animals with spinal cord damage. After administration of test compounds at specified time points, rats were separated into groups for cerebrospinal fluid sampling. Collected fractions (1.5 ml) were boiled for 12 min and centrifuged at a rate of 10,000 rpm for 10 min. The supernatant was collected, frozen at −80 °C and lyophilized. Samples were reconstituted in radioimmunoassay buffer for peptide measurement.

2.6. Measurement of spinal opioid peptides

Measurements of immunoreactive dynorphin A (1–17), leu-enkephalin and β-endorphin (pg/100 μl) in both diabetic and non-diabetic rats injected i.p. with either morphine or Δ^9-THC were accomplished using peptide-specific RIA kits obtained from Peninsula Laboratories (San Carlos, CA). The reconstituted samples were analyzed in duplicate using the cerebrospinal fluid from at least 6 individual rats for each peptide determination. Cerebrospinal fluid samples were not pooled. Only the liner portion of the RIA standard curve was used to calculate peptide concentrations. Before lyophilization, all samples consisted of 1.5 ml cerebrospinal fluid. Samples analyzed for dynorphin A were reconstituted in 250 μl RIA buffer, resulting in a 6-fold concentration of the original cerebrospinal fluid. Leucine enkephalin and β-endorphin were analyzed from samples that were reconstituted in 500 μl RIA buffer, resulting in a 3-fold concentration of cerebrospinal fluid. All peptide concentrations are reported as converted to pg/ml of cerebrospinal fluid.

2.7. Statistical analysis

Data from opioid peptide release was analyzed with Statview using analysis of variance (ANOVA) with comparisons using Dunnett's *t*-test. Data from endogenous peptide levels was further adjusted by the removal of outliers using a modified method of Dixon and Massey (1969).

3. Results

3.1. Morphine and Δ^9-THC in non-diabetic and diabetic rodents

Morphine, (2–10 mg/kg, s.c.), significantly decreased potency in streptozotocin-induced diabetic mice with an ED_{50} (+95% confidence limit) of 6.1 mg/kg (5.1–7.2) as compared to non-diabetic mice [ED_{50}=3.2 mg/kg (2.4–4.1)] (Fig. 1). Δ^9-THC has a significantly higher antinociceptive effect at a dose of 50 mg/kg in diabetic mice versus naïve mice (Fig. 2). Δ^9-THC exhibits only partial agonist effects at the cannabinoid CB_1 receptor and an ED_{50} dose could not be determined in either diabetics or non-diabetics.

In combination with Δ^9-THC (20 mg/kg, p.o.), morphine-induced (s.c.) antinociception is significantly enhanced in both

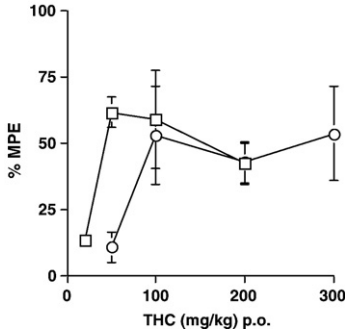

Fig. 2. The antinociceptive effects of Δ^9-THC, p.o. were evaluated in diabetic (open squares) and non-diabetic (naïve, open circles) mice by the mouse tail-flick test. Baseline tail-flick latencies were recorded and mice were given Δ^9-THC by oral gavage in doses ranging from 20–300 mg/kg, p.o., then tested in the tail-flick test 30 min post-administration to evaluate the antinociceptive effect of Δ^9-THC. In contrast to morphine, a full agonist at the mu opioid receptor, Δ^9-THC, p.o., which exhibits a partial agonist at the cannabinoid receptor CB_1 receptor activity was found to have increased antinociceptive activity at 50 mg/kg in diabetic mice as compared to naïve mice (ED_{50}=210.4 mg/kg [105.4–420.7]). The ED_{50}s could not be determined. % MPE = percent maximal effect; ED_{50} = effective dose 50%; conf. limits = 95% confidence limits; THC = Δ^9-THC 20 mg/kg, p.o.; vehicle (1:1:18) = ethanol: emulphor:saline; BG = blood glucose; V = vehicle; M = morphine; T = Δ^9-THC; D = diabetic.

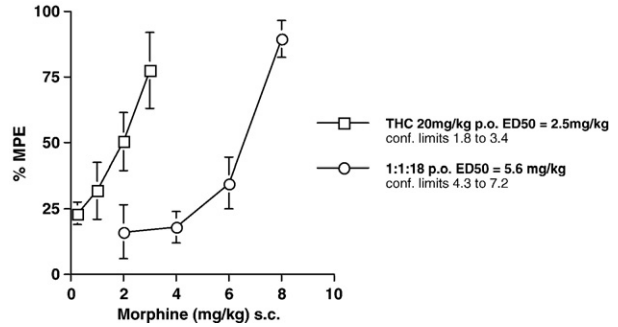

Fig. 3. A comparison of the antinociceptive effect of a combination of morphine, s.c. and Δ^9-THC (20 mg/kg, p.o.; an antinociceptively inactive dose of Δ^9-THC) to morphine (s.c.) plus vehicle (1:1:18) p.o. only in non-diabetic mice. Baseline tail-flick values were recorded, mice were given vehicle (1:1:18) p.o. or Δ^9-THC 20 mg/kg, p.o. followed 15 min by morphine in doses 0.25–8 mg/kg, s.c., and tested in the tail-flick test 15 min post-morphine administration to evaluate the antinociceptive potency of the Δ^9-THC/morphine combination. Pre-treatment with Δ^9-THC 20 mg/kg, p.o. significantly increased the antinociceptive potency of morphine s.c. in naïve mice (ED_{50}=2.5 mg/kg [1.8–3.4]) as compared to morphine with vehicle (1:1:18) (ED_{50}=5.6 mg/kg [4.3–7.2]). %MPE = percent maximal effect; ED_{50} = effective dose 50%; conf. limits = 95% confidence limits; THC = Δ^9-THC 20 mg/kg, p.o.; vehicle (1:1:18) = ethanol:emulphor:saline; BG = blood glucose; V = vehicle; M = morphine; T = Δ^9-THC; D = diabetic.

non-diabetic mice [ED_{50}=2.5 mg/kg (1.8–3.4)], as well as diabetic mice [ED_{50}=0.84 mg/kg (0.79–0.89)] (Fig. 3 and 4, respectively), although the enhancement is greatest in degree in diabetic mice (Fig. 4). The dose of Δ^9-THC (20 mg/kg, p.o.) evaluated in combination with morphine was chosen for its lack of antinociceptive activity.

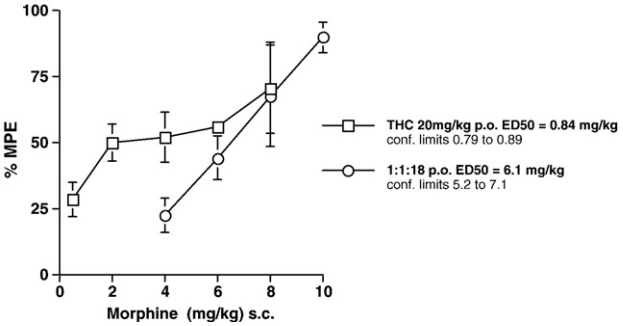

Fig. 4. A comparison of the antinociceptive effect of combined morphine, s.c. with Δ^9-THC 20 mg/kg, p.o. to morphine, s.c. with vehicle (1:1:18) p.o. only in diabetic mice. Baseline tail-flick values were recorded, diabetic mice (BG>450 mg/dl) were given vehicle (1:1:18) p.o. or Δ^9-THC 20 mg/kg, p.o. followed 15 min later by morphine in doses ranging from 0.25–8 mg/kg, s.c., then tested 15 min post-morphine administration to evaluate the antinociceptive potency of the Δ^9-THC/morphine combination as in Fig. 3. The antinociceptive potency of morphine (s.c.) in diabetic mice (ED_{50}=6.1 mg/kg [5.2–7.1]) was enhanced significantly (ED_{50}=0.84 mg/kg [0.79–0.89]) by the addition of Δ^9-THC (20 mg/kg, p.o.; an antinociceptively inactive dose of Δ^9-THC) resulting in a leftward shift in the morphine DRC as compared to morphine with vehicle (1:1:18). We observed a significant difference in ED_{50} values as noted by non-overlapping confidence limits. %MPE = percent maximal effect; ED_{50} = effective dose 50%; conf. limits = 95% confidence limits; THC = Δ^9-THC 20 mg/kg, p.o.; vehicle (1:1:18) = ethanol:emulphor:saline; BG = blood glucose; V = vehicle; M = morphine; T = Δ^9-THC; D = diabetic.

3.2. Antinociceptive effects of morphine and THC in the rat: effects of morphine on endogenous opioid release

Due to the inability to obtain adequate cerebrospinal fluid from the mouse, studies of endogenous opioid release were by necessity performed in the rat. The ED_{50} values for both morphine alone and Δ^9-THC alone were determined following the administration of morphine (i.p.) and Δ^9-THC (i.p.) in a manner similar to that described in the mice using the tail-flick test. The ED_{50} for morphine in the diabetic rat was significantly greater than that in the non-diabetic rat [5.3 (3.6–5) mg/kg versus 1.9 (1.6–3) mg/kg, respectively]. The ED_{50} for Δ^9-THC in the diabetic rat was not significantly less than that in the non-diabetic rat [1.6 (1.2–3) mg/kg versus 2.6 (1.9–4) mg/kg, respectively]. Following determination of the ED_{50} values for morphine and Δ^9-THC in non-diabetic and diabetic rats, separate groups of rats were tested for baseline values tail-flick latency in both non-diabetic and diabetic rats (Fig. 5

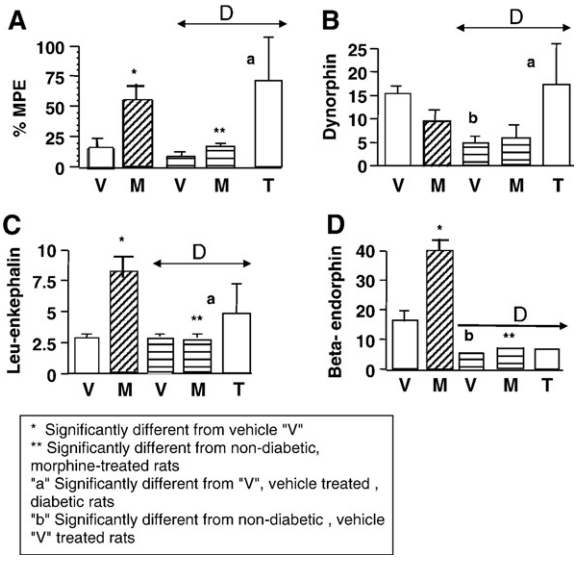

Fig. 5. Measurement of antinociceptive effects (Panel A), dynorphin A (1–17) levels (Panel B), leucine enkephalin levels (Panel C), or β-endorphin levels (Panel D) in rat cerebrospinal fluid post-administration of morphine (M) or Δ^9-THC (T) in non-diabetic or diabetic (arrow indicates bars with "D") rats. Extraction of opioid peptides was performed as described in the Methods" section. Morphine (5 mg/kg, i.p.), Δ^9-THC (2 mg/kg, i.p.) or the appropriate vehicles were injected, antinociception was quantified using the tail-flick test as previously described, and simultaneously cerebrospinal fluid was collected for analysis. Such a protocol allows for the temporal correlation between antinociception and opioid peptide release in the spinal fluid. Samples were processed and later analyzed by radioimmunoassay to determine levels of dynorphin, leu-enkephalin and β-endorphin (pg/ml). Morphine (5 mg/kg) produced a significant antinociceptive effect in non-diabetic rats (* = $p<0.05$ from "V" in non-diabetics). The antinociceptive effects of morphine were diminished in diabetic rats (** = $p<0.05$ from morphine in non-diabetic rats). Δ^9-THC (T) produced a significant antinociceptive effect in the diabetic rat ("a" = $p<0.05$). In addition, the basal levels (indicated by "V") of dynorphin A (1–17) (Panel B) and beta-endorphin (Panel D) were significantly reduced in the diabetic rat versus the non-diabetic rat ("b" = $p<0.05$). The ability of morphine to release both beta-endorphin and leucine enkephalin was significantly reduced in the diabetics. Δ^9-THC retained the ability to release leu-enkephalin and dynorphin in the diabetic rats, but did not release beta-endorphin. A more detailed description of Fig. 5 is included in the "Results" section. %MPE = percent maximal effect; ED_{50} = effective dose 50%; conf. limits = 95% confidence limits; THC = Δ^9-THC 20 mg/kg, p.o.; vehicle (1:1:18) = ethanol:emulphor:saline; BG = blood glucose; V = vehicle; M = morphine; T = Δ^9-THC; D = diabetic.

Panel A). The ED_{50} dose of morphine (5 mg/kg, i.p.) or Δ^9-THC (2 mg/kg, i.p.) in the non-diabetic rat or the appropriate vehicles were administered to either non-diabetic or diabetic rats. Antinociception was determined in the rats via the tail-flick test simultaneously with cerebrospinal fluid extraction for quantitation of endogenous opioid levels. The %MPE values were recorded at the peak time after morphine (30 min) or Δ^9-THC (30 min) administration. At least 8 rats were tested per drug. As expected, the antinociceptive effect of morphine was significantly ($p<0.05$) reduced in the diabetic rats (bars marked with "D" represent groups of diabetic rats). Δ^9-THC (ED_{50} dose in the non-diabetic rat) retained antinociceptive activity in the diabetic rats (Fig. 5 Panel A). In addition, diabetic rats had significantly lower levels of release of dynorphin and beta-endorphin in response to vehicle ("V") as compared to non-diabetic rats ($p<0.05$) (Fig. 5 Panels C and D). Morphine (ED_{50} dose of 5 mg/kg, s.c.) released significantly less leu-enkephalin in diabetic rats (Fig. 5, Panel C). Morphine released β-endorphin in non-diabetic rats, but also released significantly less β-endorphin in diabetic rats (Fig. 5, Panel D).

3.3. Δ^9-THC on endogenous opioid release

We have previously demonstrated that Δ^9-THC releases dynorphin and leu-enkephalin in non-diabetic rats (Mason et al., 1999; Cox and Welch, 2004). In the current study Δ^9-THC released endogenous opioids dynorphin A (1–17) and leu-enkephalin in diabetic rats (Fig. 5, Panels B and C, respectively). The amount of endogenous opioids, dynorphin and leucine enkephalin, released by Δ^9-THC was similar in quantity to that previously published in the non-diabetic, normal rat (Mason et al., 1999; Cox and Welch, 2004) and is thus, not shown in Fig. 5 for the normal non-diabetic animals. Δ^9-THC-induced release of dynorphin and leu-enkephalin restored the levels of these endogenous opioids to those released by morphine in non-diabetics (compare Fig. 5, Panels B and C). However, Δ^9-THC, that does not release β-endorphin in normal rats, failed to release β-endorphin to the extent observed in non-diabetics treated with morphine (Fig. 5, Panel D).

4. Discussion

Current treatment for diabetic neuropathies in humans has been extensively reviewed (Stillman, 2006; Jensen et al., 2006; Zeigler, 2006; Head, 2006; Tesfaye, 2007). Painful diabetic neuropathy is difficult to control, and current therapies such as narcotic and non-narcotic analgesics, antidepressants and anticonvulsants are often accompanied by a high risk for adverse effects (Gidal, 2006). Two of the more recently developed antiepileptic drugs, gabapentin and pregabalin, exhibit favorable pharmacokinetic and pharmacodynamic profiles, however further observation is needed to determine if pregabalin will meet previously unmet pain management needs of patients with diabetic peripheral neuropathy and other types of neuropathic pain (Sonnett et al., 2006; Bennett and Simpson, 2004; Frampton and Scott, 2004). The "bottom line" in all reviews is that the treatments available (non-steroidal antiinflammatories, steroidal antiinflammatories, antidepressants, anxiolytics and neuromuscular relaxants, as well as opioids) are often ineffective and produce adverse side effects. In addition, the decrease in efficacy and ultimate tolerance to morphine and other opioids observed in diabetics, limit the use of such drugs (Guirguis-Blake and Kelly, 2007; Ballantyne, 2006; Courteix et al., 1994; Kamei et al., 1998; Rani et al., 1996; Suh et al., 1996; Raz et al., 1988; Shook and Dewey, 1986). It is not surprising that side effects and tolerance are observed given the high and ever-escalating doses of the opioids that are required. It is our contention that if we decrease the dose of opioids administered, but maintain antinociception by the adjunct administration of Δ^9-THC, we will be able to produce long-term antinociceptive effects at doses devoid of substantial side effects.

We hypothesize that one can capitalize on the lack of toxicity of the cannabinoids and the potent analgesia of the opioids to produce a combination antinociceptive agent. In addition, although the cannabinoid and opioid receptors are located in spinal and supraspinal pain pathways [as well as in peripheral sites of pain (Calignano et al., 1998; Hohmann and Herkenham, 1998, Meng et al., 1998; Lichtman et al., 1996; Herkenham et al., 1991a; Herkenham et al., 1991b; Herkenham et al., 1991c)], they do not generally co-localize in pathways associated with many of the side effects associated with either drug alone (see review by Manzanares et al., 1999) such as medullary control of respiration by opioids, and ataxia production by cerebellar effects of cannabinoids. Thus, the use of these two classes of drugs in combination is logical in that enhancement of side effects is less likely.

In the present study, we sought to evaluate the antinociceptive efficacy of a combination of morphine and Δ^9-THC and to determine the effect of each substance alone on basal endogenous opioid levels. We chose mice as an initial screen of antinociceptive studies based upon our previous work and for the use of the well-documented mouse tail-flick latency test as a means of evaluation antinociceptive potency. When combined with Δ^9-THC, morphine-induced antinociception is significantly enhanced in both non-diabetic, as well as diabetic mice although the enhancement was found to be greatest in degree in diabetic mice. Rats were employed for the collection of cerebrospinal fluid and subsequent measuring of endogenous opioids. Morphine was found to release both leu-enkephalin and β-endorphin in non-diabetic rats, but failed to release the endogenous opioids in diabetic rats, whereas Δ^9-THC-induced released of dynorphin and leu-enkephalin was so marked it restored the levels of these endogenous opioids to those seen in morphine-treated non-diabetic rats. We did not observe the release β-endorphin by Δ^9-THC to the extent observed in non-diabetics treated with morphine. These results lead us to believe that diabetes itself may play a role in modulating the ability of morphine to release endogenous opioids and produce analgesia in diabetic models of pain.

Previous studies of endogenous opioid levels in diabetics have yielded contradictory results, although levels of various endogenous opioids appear to be altered in diabetic models. High plasma levels of [Met^5]-enkephalin (Fallucca et al., 1996; Negri et al., 1992) have been reported in patients with diabetes, whereas there is some controversy concerning levels of β-endorphin.

Vermes et al. (1985) observed normal levels of β-endorphin in diabetic patients, however, Cheung and Tang (1999) observed a decrease in β-endorphin levels in the rat neuro-intermediate pituitary compared to elevated levels in the anterior pituitary, as well as inhibition of pituitary synthesis of its precursor proopiomelanocortin (POMC). Elevated levels of [Met^5]-enkephalin have also been recorded in genetically diabetic (db/db) mice (Greenberg et al., 1985; Timmers et al., 1986), and prodynorphin peptides have been reported to be elevated in the brain of diabetic rats (Berman et al., 1995). Tsigos et al. (1995) found that β-endorphin levels in cerebrospinal fluid appear to be reduced in diabetic polyneuropathy, but found no relation to the presence of neuropathic pain, as the levels of β-endorphin were similar in neuropathic subjects with and without pain. The direct quantitation of endogenous opioid levels in diabetic rat spinal cord or cerebrospinal fluid has not been previously published. Since exogenous opioid agonists rely in part upon the release of endogenous opioids for their antinociceptive effects, a decrease in releasable endogenous opioids in diabetic animals might explain why opioid analgesics are less effective in treating painful diabetic neuropathy. Conversely, a drug that would "normalize" resting endogenous opioid tone might be expected to produce an antihyperalgesic effect. This is further supported by the favorable response of patients with neurogenic pain, compared to those with other chronic pain conditions, to acupuncture and transcutaneous electrical nerve stimulation that have been shown to increase the activity of the opioid peptide systems (Han and Terenius, 1984).

Although several hypotheses exist (including that addressed in this paper), clearly the mechanisms underlying decreased analgesic potency of opioids such as morphine in diabetic neuropathic pain and in diabetic animals with acute pain are not fully known. Chen and Pan (2003) found that despite the decrease in the antinociceptive effect of morphine in diabetic rats, the number of mu opioid receptors was similar in normal and diabetic rats. These findings led to the prediction that the reduced antinociceptive effect may be due to an impairment of mu opioid receptor/G-protein coupling since G-protein activation by mu opioid receptor agonists was significantly reduced in the spinal cord dorsal horn in diabetic animals. However, there is evidence for the involvement of the hyperglycemic component of diabetes in the initial reduction in antinociceptive activity of morphine and other opioid analgesics (Simon and Dewey, 1981). Administration of insulin to streptozotocin-induced diabetic mice, which normalizes serum glucose levels, restores sensitivity to the antinociceptive effects of morphine. There is also evidence of a glucose-induced alteration of opioid receptor affinity (Brase et al., 1987). Brase et al. (1987) demonstrated that high-affinity opioid binding sites displayed concentration-dependent decreases in affinity, but no significant affect on maximum number of binding sites, with increasing concentrations of glucose in vitro.

Thus, although studies have predicted several hypotheses for the reduced antinociceptive effect of morphine in diabetic models, morphine and its derivatives do not produce adequate analgesia in this disease state and alternative treatments are required for the treatment of pain. We have previously shown that morphine and Δ^9-THC exhibit synergy in the expression of antinociception in both acute and chronic pain models (Cichewicz and McCarthy, 2003; Cox et al., 2007a), and have extended our data to include acute pain in diabetic rats and mice. Because opioid receptors are activated physiologically by the products of endogenous opioid peptide genes, proenkephalin and prodynorphin, exogenous opioids rely in part on the release of endogenous opioids for their antinociceptive actions (Pugh et al., 1996). Our data point strongly to the induction of diabetes producing a reduced endogenous opioid tone, possibly leading to enhanced sensitivity to painful stimuli. Diabetic rats have significantly lower basal levels of endogenous opioids β-endorphin and dynorphin A (1–17). Morphine is less potent in diabetic mice (Gul et al., 2000; Ohsawa et al., 1998; Ohsawa et al., 2000; Simon and Dewey, 1981) and rats (Chen et al., 2002) than naïve subjects, an effect that may be due to the lack of release of beta-endorphin or leu-enkephalin in diabetic rats. Though the mechanism underlying the decrease in potency of opioids in diabetic models is not clear, strong evidence supports that the attenuation is caused by alterations in second and/or third messengers or possibly ion channels further downstream. Ohsawa et al. (2000) demonstrated that in streptozotocin-induced diabetic mice, the effect of mu opioid receptor agonist DAMGO was significantly attenuated as compared to that in non-diabetic mice. Because they did not observe any differences in the mu opioid receptor-mediated G-protein activation between non-diabetic and diabetic mice, they concluded that this attenuation was likely caused by a dysfunction in the cellular pathways downstream of the activation of G-proteins. There is evidence that there is bidirectional cross-talk between the insulin receptor and G-protein coupled receptors such as mu opioid receptors (Li et al., 2003). Li et al. (2003) showed that morphine stimulates serine phosphorylation of insulin receptors, resulting in disruption of functional signaling complexes that couple the insulin response to the ERK and Akt pathway. Conversely, the association of the insulin receptors with appropriate signaling molecules and subsequent recruitment of downstream signaling molecules leads to activation of the ERK pathway, which results in gene expression changes which initiate a response to cannabinoids (Li et al., 2003). Although this response is not completely understood, the proposed neuromodulatory function of Δ^9-THC may explain its increased activity in diabetic models of pain.

The effect of hyperglycemia on nociception further complicates the role of endogenous opioids in pain states. As mentioned, the hyperglycemic aspect of diabetes appears to underlie at least in part the reduced antinociceptive effects of morphine and other opioid analgesics in diabetic subjects (Simon and Dewey, 1981). Su et al. (2005) has shown an association between recovery of insulin sensitivity in obese Zucker rats by regular exercise and recovery of levels of endogenous β-endorphin. These findings, consistent with our findings of a decreased level of β-endorphin in diabetics, were consistent with previous observations that insulin resistance develops more quickly in the absence of mu opioid receptors (Guillemin et al., 1977), and the fact that exercise increases levels of β-endorphin.

In summary, the effects of Δ^9-THC on endogenous opioid modulation depend upon basal endogenous opioid tone, and it is hypothesized (Cox and Welch, 2004) that Δ^9-THC modulates endogenous opioid tone. When endogenous opioid levels are

low, Δ^9-THC increases them and conversely when endogenous opioids levels are high, Δ^9-THC decreases them (Cox and Welch, 2004). Because, there are differences between basal endogenous opioid tone in arthritis versus diabetic neuropathy, the mechanisms by which Δ^9-THC induces antinociception and enhances opioids likely differs in each condition. The activity of Δ^9-THC in chronic pain appears to be mediated by both cannabinoid CB_1 and cannabinoid CB_2 receptor activation; whereas acute pain appears to be mediated by the cannabinoid CB_1 receptor only (Cox et al., 2007b). WIN 55,212-2, a mixed CB1 and CB2 cannabinoid receptor agonist, has been shown to be effective against hyperalgesia and allodynia (Ulugol et al., 2004; Bridges et al., 2001; Herzberg et al., 1997). Neuropathic pain is that which is characterized by spontaneous pain; hyperalgesia or an augmented pain response to normally painful stimuli; and allodynia, which is a nociceptive response to normally innocuous stimuli (Ulugol et al., 2004). Diabetes may, like peripheral nerve injury models, also lead to neuropathic pain. The tail-flick test is commonly used to measure spinally-mediated pain as the result of a thermal stimulus. Thus, the tail-flick test and diabetic neuropathy (allodynia) are very different types of pain and tail-flick results may not necessarily generalize to allodynia. These studies might be extended to an alternative pharmacotherapy regimen for the treatment of pain associated with diabetic neuropathy, although such work was not the subject of these studies.

In addition, although not the subject of this study, endocannabinoid tone and the modulation thereof has been recently reviewed (Degroot and Nomikos, 2007; Maione et al., 2006) indicating a significant role for the endocannabinoid system in various chronic pain states (Palazzo et al., 2006; La Rana et al., 2006; Petrosino et al., 2007). In addition, the use of inhibitors of the degradation of the endocannabinoids by fatty acid amide hydrolase (FAAH) has been shown to decrease both acute (Wallace et al., 2007; Haller et al., 2006; Jhaveri et al., 2006; Piomelli et al., 2006) and chronic pain (Costa et al., 2006; Russo et al., 2007; Jayamanne et al., 2006) via either cannabinoid CB1 or cannabinoid CB2 or a proposed novel non-cannabinoid CB1/CB2 receptor. Our results using Δ^9-THC may clearly differ from those observed with endocannabinoids. However, our results indicate the potential for the activation of the cannabinoid receptor, by exogenous or endogenous cannabinoids or FAAH inhibitors resulting in profound effects on endogenous opioid basal levels, release, and subsequent production of antinociception in a diabetic model, alone or in combination with low doses of morphine. The clinical utility of such combinations has yet to be studied, but has the possibility of producing effective pain relief with a decrease in side effects, not the least of which is tolerance to both cannabinoids and opioids as we have previously demonstrated in acute pain models utilizing the drugs in combination (Cichewicz and Welch, 2003).

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