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Repeated Cannabinoid Injections into the Rat Periaqueductal Gray Enhances Subsequent Morphine Antinociception

Adrianne R. Wilson[a], **Lauren Maher**, and **Michael M. Morgan**[a,b]

[a]Department of Neuroscience, Washington State University Vancouver

[b]Department of Psychology, Washington State University Vancouver

SUMMARY

Cannabinoids and opiates inhibit pain, in part, by activating the periaqueductal gray (PAG). Evidence suggests this activation occurs through distinct mechanisms. If the antinociceptive mechanisms are distinct, then cross-tolerance between opioids and cannabinoids should not develop. This hypothesis was tested by measuring the antinociceptive effect of microinjecting morphine into the ventrolateral PAG of rats pretreated with the cannabinoid HU-210 for two days. Male Spraque-Dawley rats were injected twice a day for two days with vehicle (0.4 μL), morphine (5μg/0.4 μL), HU-210 (5μg/0.4 μL), or morphine combined with HU-210 into the ventrolateral PAG. Repeated injections of morphine caused a rightward shift in the morphine dose response curve on Day 3 (i.e., tolerance developed). No tolerance was evident in rats pretreated with morphine combined with HU-210. In rats pretreated with HU-210 alone, morphine antinociception was enhanced. This enhancement was blocked by pretreating rats with the cannabinoid receptor antagonist AM-251, and it also disappeared when rats were tested one week later. Acute microinjection of HU-210 into the PAG antagonized morphine antinociception, suggesting that HU-210-induced enhancement of morphine antinociception is a compensatory response. As hypothesized, there was no evidence of cross-tolerance between morphine and HU-210. In fact, cannabinoid pretreatment enhanced the antinociceptive effect of microinjecting morphine into the ventrolateral PAG. These findings suggest that alternating opioid and cannabinoid treatment could be therapeutically advantageous by preventing the development of tolerance and enhancing morphine antinociception.

Keywords

pain; analgesia; tolerance; cross-tolerance; opioid; opiate

INTRODUCTION

Opiates such as morphine are the most effective treatment for severe pain. Morphine produces antinociception in part by activating the descending pain modulatory system that projects from the periaqueductal grey (PAG) to the rostral ventromedial medulla (RVM) to the dorsal horn of the spinal cord (Basbaum and Fields, 1984). Morphine microinjection into the PAG is

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Address for correspondence: Michael Morgan, 14204 NE Salmon Creek Avenue, Vancouver, Washington 98686, morgan@vancouver.wsu.edu, Phone: (360)546-9742, Fax: (360)546-9038.

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sufficient to produce antinociception (Jensen and Yaksh, 1986; Morgan et al., 1998), and blocking opioids in the PAG is sufficient to attenuate the antinociceptive effect of systemic morphine administration (Bernal et al., 2007; Lane et al., 2005; Randich et al., 1992; Zambotti et al., 1982).

The ventrolateral region of the PAG has been shown to play an important role in the development of tolerance to the antinociceptive effects of morphine. Tolerance develops to repeated microinjections of morphine into the ventrolateral PAG (Jacquet and Lajtha, 1974; Morgan et al., 2006a; Siuciak and Advokat, 1987; Tortorici et al., 1999) and blocking opioid receptors in the ventrolateral PAG attenuates tolerance to systemic morphine administration (Lane et al., 2005).

One way to limit the development of tolerance is to limit the duration of drug administration (Suzuki et al., 1983). This can be accomplished while maintaining pain treatment by alternating administration of different drugs. Given that microinjection of cannabinoid agonists into the PAG produces antinociception (Lichtman et al., 1996; Martin et al., 1995; Martin et al., 1998; Meng and Johansen, 2004; Welch and Stevens, 1992), alternating administration of a cannabinoid agonist and morphine could maintain the potency of both drugs. Of course, this is possible only if cross-tolerance does not develop from cannabinoids to morphine. The synergistic antinociception produced by systemic administration of cannabinoids and morphine suggests that these drugs produce antinociception through different mechanisms (Cichewicz and McCarthy, 2003; Roberts et al., 2006; Tham et al., 2005). The lack of cross-tolerance between opioids and cannabinoids after intrathecal or systemic administration (Mao et al., 2000; Yesilyurt and Dogrul, 2004) is consistent with this hypothesis. The objective of the present study was to test this hypothesis in the PAG by examining changes in morphine potency following repeated microinjections of the cannabinoid receptor agonist HU-210.

METHODS

Subjects

Male Sprague-Dawley rats (212–361 g; Harlan, Kent WA) were anesthetized with equithesin (60 mg/kg, i.p.) and stereotaxically implanted with a 23-gauge (.573 mm) stainless steel guide cannula aimed at the ventrolateral PAG (9 mm long, AP +2.3 mm; ML −0.6 mm; DV −4.6 mm from lambda). The guide cannula was held in place with dental cement affixed to two screws in the skull. Following surgery, a removable stainless steel stylet was inserted into the guide cannula, and the rat was housed individually with food and water available *ad libitum*. Lights were maintained on a reverse 12 h light/dark cycle so that all injections and testing occurred during the animals' active phase, rather than during the light, inactive phase.

Rats were handled daily prior to and following surgery. Testing began one week following surgery. Animals were not re-used for subsequent experiments, nor were they pretreated with more than one drug or drug combination. Experiments were conducted in accordance with the animal care and use guidelines of the International Association for the Study of Pain. The Institutional Animal Care and Use Committee at Washington State University approved this research.

Microinjections

The day before the first microinjection, an 11 mm injection cannula was inserted into the guide cannula without administration of drugs. This procedure reduces artifacts on the test day resulting from mechanical damage to neurons and habituates rats to the injection procedure.

Microinjections were administered through a 31 gauge (.226 mm), 11 mm long injection cannula inserted into and extending 2 mm beyond the tip of the 9 mm guide cannula. The

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injection cannula was connected to a 1 µl syringe (Hamilton Co., Reno, NV) with PE20 tubing filled with sterile water. All microinjections were administered in a volume of 0.4 µL over 40 s while the rat was gently restrained. The injection cannula remained in place an additional 20 s to minimize backflow of the drug up the cannula tract. Following the injection, the stylet was reinserted into the guide cannula and the rat was returned to its home cage.

Although repeated microinjections may cause some localized damage, this is inevitable in any microinjection study. Microinjections administered outside of the target area were used as a control for the effects of repeated microinjections.

Drugs

Drug doses and concentrations were selected based on previous studies (Finn et al., 2003; Morgan et al., 2005). For pretreatment procedures, morphine sulfate (a gift from the National Institute on Drug Abuse), the cannabinoid receptor agonist HU-210 (Tocris, St. Louis, MO), and the cannabinoid receptor antagonist AM-251 (Tocris) were dissolved in 60% DMSO and saline. The control groups received microinjections of vehicle (60% DMSO in saline). Morphine was dissolved in saline for the cumulative dose assessment of morphine potency (Morgan et al.2006). Rats receiving morphine and HU-210 received both drugs in a single injection. Each rat was used for only one experiment, and all rats in each experiment received the same number of microinjections, regardless of treatment. In Experiments 1 and 5, animals were given four injections of 0.4µL. In Experiments 2, 3, and 4, animals were given four injections of 0.4µL during pre-treatment, and five subsequent 0.4µL injections to assess morphine dose response characteristics. For the HU-210 cumulative dosing procedure, actual doses injected were 3.2, 2.4, 4.4, and 8.0µg, resulting in cumulative quarter log doses of 3.2, 5.6, 10.0, and 18µg. For the morphine cumulative dosing procedure, actual doses injected were 1.0, 0.8, 1.4, 2.4, and 4.4µg, resulting in cumulative quarter log doses of 1.0, 1.8, 3.2, 5.6, and 10µg.

Behavioral Tests

Nociception was assessed using the hot plate test because this test can be used repeatedly and is sensitive to morphine antinociception (Morgan et al., 2006b). The hot plate test measures the latency for a rat to lick a hind paw when placed on a 52.5°C surface. The rat was immediately removed from the hot plate following a response, or after 40 s if no response occurred. Hot plate calibration at this temperature produced baseline latencies of 9 to 14 s. Given that microinjection of morphine into the ventrolateral PAG causes circling or immobility (Morgan et al., 1998), locomotion was assessed by placing the rat in an open field (1 × 0.6 m) and counting the number of squares (15 × 15 cm) entered by the forepaws in 30 s. The experimenter conducting the behavioral tests was blind to the pretreatment condition of the animals.

Experiment 1: Acute HU-210 PAG Microinjections

The antinociceptive effect of microinjecting cumulative doses of HU-210 into the ventrolateral PAG was assessed (N = 10). Microinjections were administered every 20 min in cumulative quarter log doses of 3.2, 5.6, 10.0, & 18.0 µg/0.4 µL. In six rats, the highest injection administered was 14.4 µg, not 18.0 µg. Following baseline measurements, hot plate and open field tests were conducted 15 min after each microinjection of HU-210.

Experiment 2: Morphine/HU-210 Cross-Tolerance

Rats were injected with morphine (5 µg/0.4 µL, N = 8), HU-210 (5 µg/0.4 µL, N = 6), 60% DMSO vehicle (N = 6), or a morphine/HU-210 combination (N = 7) twice a day (0930 & 1600) for two days (Trials 1 – 4). Thirty minutes after the first injection on Day 1, nociception was assessed using the hot plate test. No testing was conducted following injections on Trials 2–4

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to limit changes in latency caused by repeated testing (Gamble and Milne, 1989; Lane et al., 2004).

On Day 3, tolerance to morphine was assessed using cumulative dose microinjections (1.0, 1.8, 3.2, 5.6, and 10 µg/0.4 µL) into the ventrolateral PAG at 20 min intervals (Morgan et al., 2006a). Nociception and open field activity were assessed 15 min after each microinjection. This procedure was applied consistently in all subsequent experiments where the cumulative morphine dosing procedure was used.

Experiment 3: Cannabinoid Antagonism

This experiment was conducted to determine whether the effect of HU-210 on morphine antinociception was mediated by cannabinoid receptors. Rats were injected intraperitoneally with either the cannabinoid antagonist AM-251 (1 mg/kg, N = 14) or an equivalent volume of 60% DMSO vehicle (N = 8). HU-210 (5 µg/0.4 µL) was microinjected into the PAG fifteen minutes later. This procedure was repeated twice a day for two days (Trials 1–4). Nociception was assessed using the hot plate test thirty minutes after microinjection of HU-210 on Trial 1. No testing was conducted on Trials 2–4. On Day 3 (Trial 5) morphine antinociception was assessed using the cumulative microinjection procedure described in Experiment 2.

Experiment 4: Duration of HU-210 Induced Enhancement

The objective of this experiment was to determine the duration of changes produced by repeated microinjection of HU-210 into the ventrolateral PAG. The experimental procedure was identical to Experiment 2 except that rats were tested on Day 8 instead of Day 3. Rats were pretreated with ventrolateral PAG microinjections of morphine (N = 6), HU-210 (N = 5), 60% DMSO vehicle (N = 7), or a morphine/HU-210 combination (N = 5) twice a day for two days (Trials 1 – 4). No drug administration or behavioral testing were conducted on Days 3–7.

Experiment 5: Acute HU-210 and Morphine Interaction

If the effects of HU-210 pretreatment on morphine antinociception are caused by residual HU-210 in the PAG, then acute microinjection of HU-210 into the ventrolateral PAG should have similar effects. Cumulative dose microinjections of morphine into the ventrolateral PAG were performed as described in Experiment 2 (1.0, 1.8, 3.2, 5.6, and 10 µg/0.4 µL). Half of the animals (N = 16) received 5 µg HU-210 dissolved in the first morphine microinjection (1.0 µg/0.4 µL). The vehicle for the first injection was 60% DMSO in both groups of animals, with subsequent morphine doses dissolved in saline. Injections were 20 minutes apart and nociception and open field activity were assessed 15 min after each injection.

Histology

Rats were euthanized following testing by administering a lethal dose of Halothane. The injection site was marked by microinjecting Cresyl Violet (0.2 µL) into the PAG, and the brain was removed and placed in formalin (10%). At least 2 days later the brain was sectioned coronally (100 µm) and the location of the injection site identified using the atlas of Paxinos and Watson (2005).

Data Analysis

Dose-response curves were generated for hot plate data using nonlinear regression (Graph Pad Prism). The half-maximal effective dose (D_{50}) was calculated for each condition (Tallarida, 2000). Analysis of variance (ANOVA) was used to assess changes in hot plate and open field activity (SPSS). D_{50} values calculated from dose response curves (GraphPad) and ANOVA (Tallarida, 2000) were used to assess changes in potency. Post hoc comparisons were made

with 95% confidence intervals. Statistical significance was defined as a probability of less than .05.

RESULTS

Data were derived from rats with microinjection placements in or along the border of the ventrolateral PAG (Figure 1). Placements outside the ventrolateral PAG were used as negative controls, allowing for comparisons to on-site data.

Experiment 1: Acute HU-210 PAG Microinjections

Microinjection of HU-210 (3.2, 5.6, 10.0, & 18.0 µg/0.4 µL) into the ventrolateral PAG caused a small increase in hot plate latency. Microinjection of the highest dose of HU-210 (14.4 or 18.0 µg/0.4 µL) into the ventrolateral PAG resulted in a 59% increase in hot plate latency compared to baseline (9.5 ± 0.8 vs. 15.1 ± 1.1 s; $F(4,45) = 4.7$, $p < .05$). This increase is modest compared to morphine antinociception (Morgan et al., 2006a), but comparable to the antinociception reported in previous PAG microinjection studies (Finn et al., 2003; Lichtman et al., 1996). Also in concordance with previous reports, there was no dose-dependent effect of HU-210 on locomotor behavior (Finn et al., 2003). Mean open field activity ranged from 13 to squares entered following administration of the various HU-210 doses.

Experiment 2: Morphine/HU-210 Cross-Tolerance

Microinjection of HU-210 (5 µg/0.4 µL) into the ventrolateral PAG did not cause a statistically significant increase in hot plate latency compared to vehicle treated rats on Trial 1 ($F(1,23) = 3.1$, $p > .05$). In contrast, microinjection of morphine (5 µg/0.4 µL) into the ventrolateral PAG caused a significant increase in hot plate latency to 33.1 s ± 4.0 s compared to 14.9 s ± 0.95 s for vehicle treated controls ($F(1,23) = 42.3$, $p < .05$). The morphine/HU-210 group showed the greatest increase in hot plate latency (38.9s ± 1.1 s), although this antinociception did not differ significantly from rats treated with morphine alone.

Mean locomotor activity on Trial 1 did not differ between morphine (20.8 ± 6.5 squares entered), HU-210 (21.7 ± 2.4), or vehicle-pretreated animals (27.0 ± 1.8; $F(3,23) = 2.1$, $p > .05$). Combined administration of HU-210 and morphine produced the greatest decrease in locomotor activity compared to vehicle-injected animals (9.9 ± 5.1), but this effect also failed to reach statistical significance (Tukey's, $p = .10$)

Tolerance and cross-tolerance to ventrolateral PAG morphine microinjection was assessed on Trial 5. Rats pretreated with morphine showed a rightward shift in the morphine dose response curve, as would be expected with the development of tolerance ($F(3,127) = 4.1$, $p < .05$, Figure 2A). The D_{50} value for rats pretreated with vehicle was outside the 95% confidence interval of the rats pretreated with HU-210, indicating that HU-210-pretreated rats had greater morphine antinociception on Trial 5 compared to vehicle-pretreated rats (Figure 2A, Table 1). HU-210 microinjections outside the vlPAG (N = 4) did not display this enhanced morphine antinociception (mean hot plate latencies ranged from 12.6 to 20.7 s following the cumulative morphine microinjection procedure). Co-administration of HU-210 and morphine on Trials 1 – 4 blocked tolerance to morphine (Figure 2B). The D_{50} value for the combined morphine/HU-210 group value did not differ significantly from the vehicle-pretreated group when tested with morphine on Trial 5 (Table 1), despite showing maximal antinociception on Trial 1.

Microinjection of morphine into the ventrolateral PAG caused circling in some rats (N = 9), immobility in a few (N = 4) and no effect in others (N = 14), as previously reported (Morgan et al., 1998). These locomotor effects occurred equally in morphine-treated and in HU-210/morphine treated animals. Previous reports of explosive flight reactions to dorsal-lateral PAG

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morphine injections (Morgan et al. 1998) were not seen in this study, as both on and offsite injections were outside of the dorsal-lateral PAG. There was no significant difference in locomotor activity between any of the groups at any dose ($F(3,23) = 0.3$, $p = 0.83$). Morphine administration produced a dose-dependant decrease in locomotor activity in all four groups (Figure 3).

Experiment 3: Cannabinoid Antagonism

In this experiment, we assessed morphine antinociception after pretreatment with systemic AM-251 (cannabinoid antagonist) and intracranial HU-210. Mean baseline hot plate latencies were similar in rats pretreated with HU-210 (15.6 ± 1.3 s) and HU-210/AM-251 (14.8 ± 1.0 s). Pretreatment with AM-251 and blocked the leftward shift in the morphine dose response curve produced by HU-210 pretreatment (Figure 4; $F(1,106) = 3.95$, $p < .05$, Figure 4). The morphine D_{50} for animals pretreated with HU-210 was 1.53 μg (C.I. = 0.70 – 2.35), whereas the D_{50} for animals pretreated with i.p. AM-251 prior to microinjection of HU-210 was 4.32 μg (C.I. = 1.87 – 6.77).

Experiment 4: Duration of HU-210 Induced Enhancement

The longevity of the HU-210 enhancement of morphine antinociception was assessed by testing rats 6 days after termination of HU-210 pretreatment (on Day 8). Baseline hot plate latencies on Day 8 did not differ between the four pretreatment groups (ranged from 12.2 to 15.9 s). Repeated administration of morphine on Trials 1 – 4 produced tolerance to morphine on Day 8, as the morphine D_{50} value fell outside of the vehicle confidence interval (Table 1). The D_{50} values for the other pretreatment groups fell within with the vehicle confidence interval (Table 1; $F(3,107) = 0.6$, $p > .05$). Unlike Experiment 2, morphine potency in rats pretreated with HU-210 alone did not differ from vehicle-pretreated animals (Figure 5).

Experiment 5: Acute HU-210 and Acute Morphine

The enhanced morphine antinociception produced by HU-210 pretreatment could be caused by residual HU-210 in the PAG. To test this hypothesis, the acute effect of HU-210 microinjection on morphine antinociception was assessed. HU-210 (5 μg) was administered with the first of 5 cumulative morphine microinjections. Co-administration of HU-210 and morphine caused a reduction of maximal antinociception compared to animals microinjected with morphine alone (Figure 6). This difference was statistically significant as revealed by a one-tailed t-test ($t(15) = 1.77$, $p < .05$).

DISCUSSION

These experiments show that co-administration of HU-210 and morphine prevented the development of tolerance to morphine's antinociceptive effect, and of greatest interest, HU-210 pretreatment enhanced subsequent morphine antinociception. This enhancement of antinociception was prevented by AM-251 administration, and the enhancement also disappeared when animals were tested 6 days after HU-210 pretreatment. In addition, this enhanced antinociception is not caused by residual HU-210 in the PAG because acute co-administration of HU-210 and morphine diminished morphine's antinociceptive effect.

Although opioids and cannabinoids activate the same descending nociceptive modulatory system (Meng et al., 1998), evidence suggests they trigger this system through separate and distinct mechanisms. First, when co-administered systemically, delta-9-tetrahydrocannabinol (THC) and morphine produce synergistic antinociception (Cichewicz and McCarthy, 2003; Tham et al., 2005). Second, administration of the opioid receptor antagonist naloxone blocks the antinociceptive effect of morphine, but not that of cannabinoids (Bloom and Dewey, 1978; Massi et al., 2001; Meng et al., 1998; Welch, 1993). Third, unlike μ-opioids, the

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cannabinoid receptor agonist WIN55,212-2 has no effect on isolated PAG cells, yet in intact slice preparations WIN55,212-2 can inhibit evoked IPSPs and EPSPs (Vaughan et al., 2000). This difference indicates that while opioids can directly trigger post-synaptic events, cannabinoids modulate neural activity through pre-synaptic inhibition. The lack of cross-tolerance between HU-210 and morphine repeated here is consistent with these differences.

The use of marijuana as a therapeutic pain management tool has generated a great deal of publicity and controversy. THC, the primary psychoactive compound in marijuana, has been found to be effective in pain management (Corey, 2005; Walker et al., 1999; Walker et al., 2001). Cannabinoid antinociception can be produced in rats through a wide range of administration techniques, including oral, subcutaneous, intrathecal, intraperitoneal, intracerebral, and topical (Bloom and Dewey, 1978; Cichewicz et al., 1999; Dogrul et al., 2003; Hohmann, 2002; Hohmann et al., 1999a, Hohmann et al., 1999b; Lichtman et al., 1996; Manzanares et al., 1998; Welch and Stevens, 1992). The PAG also appears to contribute to the antinociceptive effects of cannabinoids. CB1 and CB2 receptors are localized in the PAG (Cristino et al., 2006; Gong et al., 2006; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998), and microinjection of cannabinoids into the PAG produces antinociception (Lichtman et al., 1996; Martin et al., 1995; Martin et al., 1998; Meng and Johansen, 2004; Welch and Stevens, 1992). In addition, microinjection of cannabinoid receptor antagonists into the PAG attenuates stress-induced antinociception (Hohmann et al., 2005).

In the current study, microinjection of HU-210 into the ventrolateral PAG caused a small increase in hot plate latency compared to that produced by microinjection of morphine. These data are consistent with previous research showing that HU-210 is more effective in producing antinociception when injected into the dorsal, compared to the ventral PAG (Martin et al. 1995). Our finding that acute co-administration of HU-210 and morphine into the PAG attenuates morphine antinociception may suggest that opioids and cannabinoids could have opposing effects within the PAG.

The PAG not only plays a significant role in opioid-mediated antinociception, but it also contributes to the development of tolerance to morphine's antinociceptive effect. Repeated microinjection of morphine directly into the ventrolateral PAG is sufficient to produce tolerance to the drug's antinociceptive effects (Morgan et al., 2006a; Tortorici et al., 1999), a finding confirmed here. Moreover, inactivation of the opioid receptors in the PAG attenuates the development of tolerance to systemic morphine administration (Lane et al., 2005). These findings indicate that the PAG is both sufficient and necessary for the development of tolerance to morphine. This tolerance appears to be mediated by opioid sensitive GABAergic neurons because direct repeated activation of ventrolateral PAG output neurons does not produce tolerance (Morgan et al., 2003). *In vitro* electrophysiological recordings from PAG neurons derived from rats pretreated with morphine show an upregulation of adenylyl cyclase in these GABAergic neurons (Garzon et al., 2005; Ingram et al., 1998).

In the present study, PAG-mediated morphine tolerance was prevented by pretreatment with the cannabinoid agonist HU-210. The morphine antinociception produced in animals pretreated with HU-210/morphine was no different than the antinociception produced in vehicle-pretreated controls. This reversal of tolerance is surprising because combined microinjection of HU-210 and morphine produced the greatest antinociception on Trial 1. This phenomenon is further substantiated by recent findings demonstrating similar attenuation of morphine tolerance by THC (Smith et al., 2007).

The cannabinoid antagonist AM-251 blocked HU-210's enhancement of antinociception, indicating that HU-210 enhances morphine antinociception via the CB1 receptor, rather than a nonspecific action of microinjecting HU-210 into the PAG. The CB1 antagonist was

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administered systemically, and could therefore act at many sites in addition to the PAG. However, because morphine and HU-210 were administered directly into the PAG, it is unlikely that any site outside of the PAG mediated this effect. It is also unlikely that residual cannabinoids cause the increased antinociception we observe on Day 3, because this would require residual HU-210 to remain in the brain 16 hours after the last microinjection. Moreover, acute HU-210 administration attenuated, rather than enhanced morphine-induced antinociception in Experiment 5.

The effect of HU-210 on antinociception is no longer present by Day 8. The mechanism by which cannabinoid pretreatment enhances antinociception in the short term (i.e., 16 hrs after administration), but not in the long term is not clear. However, this loss of effect is consistent with the loss of tolerance to PAG morphine antinociception that occurs between one and two weeks after morphine administration (Morgan et al. 2005). The exact time course of HU-210 enhancement of morphine antinociception is unknown.

In contrast to our data from the ventrolateral PAG, blockade of the cannabinoid system in the spinal cord has been shown to prevent the development of tolerance to morphine (Trang et al., 2007). This difference between the spinal cord and ventrolateral PAG could be caused by different experimental procedures or differences in the mechanism for tolerance at the two sites. Previous studies examining tolerance suggest that the mechanisms for tolerance differ between the ventrolateral PAG and spinal cord. NMDA receptor ntagonists disrupt morphine tolerance when applied at the spinal level (Trujillo and Akil, 1991), but not when microinjected into the PAG (Morgan et al., in preparation). Intrathecal co-administration of the mu-opioid receptor agonist DAMGO and morphine prevents morphine tolerance (He et al., 2002). In the PAG however, co-administration not only results in tolerance to DAMGO's antinociceptive effect, but DAMGO enhances tolerance to morphine (Meyer et al., 2007). Taken together, these results indicate that morphine tolerance differs at the spinal and supraspinal levels.

Despite the differences in tolerance between the spinal cord and the PAG, it is noteworthy and clinically relevant that systemic administration of opioids and cannabinoids show antinociceptive enhancement in a manner similar to that found in the present study. That is, morphine antinociception is greater in animals exposed to cannabinoids (Cichewicz et al., 1999; Cichewicz et al., 2005; Smith et al., 1998; Yesilyurt et al., 2003). Our data demonstrate the enhancement of morphine antinociception following HU-210 pretreatment, but not when the drugs are co-administered acutely. In addition, cannabinoid antinociception is greater in morphine-tolerant animals than in morphine-naïve animals (Cichewicz and Welch, 2003; Rubino et al., 1997; Smith et al., 2007; Vigano et al., 2005). Thus, it appears that antinociceptive enhancement between cannabinoids and opioids is bidirectional. This bidirectionality of enhanced antinociception may be partially explained by the reciprocal upregulation of CB1 and mu-opioid receptors after chronic exposure to opioids or cannabinoids respectively (Fattore et al., 2007). Although the PAG was not examined specifically, this receptor upregulation occurs in a wide range of brain areas and includes enhanced receptor efficacy. Similar changes appear to occur in the PAG as indicated by an increase in μ-opioid receptor binding and proenkephalin mRNA following systemic administration of the cannabinoid agonist CP-55,940 (Manzanares et al., 1998; Vigano et al., 2005).

Despite these findings, interactions between systemically administered cannabinoids and opioids have been shown to vary widely. Some studies show cross-tolerance between cannabinoids and opioids (Massi et al., 2001; Thorat and Bhargava, 1994), while other studies failed to find such an effect (Valverde et al., 2001; Yesilyurt and Dogrul, 2004). Such differences might be attributed to the different methods of administration utilized (e.g., topical vs. intraperitoneal) or differences between cannabinoid agonists. Conversely, and consistent with our results, some investigators show that co-administration of a cannabinoid and morphine

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blocks the development of tolerance to morphine (Cichewicz and McCarthy, 2003). Also, as mentioned previously, cannabinoid antinociception is enhanced in morphine tolerant animals (Cichewicz and Welch, 2003; Rubino et al., 1997; Vigano et al., 2005).

Administering low morphine doses for short periods of time may attenuate the negative attributes associated with morphine use. The side effects that accompany cannabis and other cannabinoid therapies, such as nabilone and dronabinol, are less severe than that produced by opioids, yet the analgesia they provide is minimal compared to opioids. Modern clinicians face the challenge of finding a treatment protocol that leads to maximal pain relief and limited side effects. The present data showed no enhancement of motor inhibition produced by morphine in rats pretreated with HU-210, suggesting that HU-210 enhancement of morphine may be specific to antinociception.

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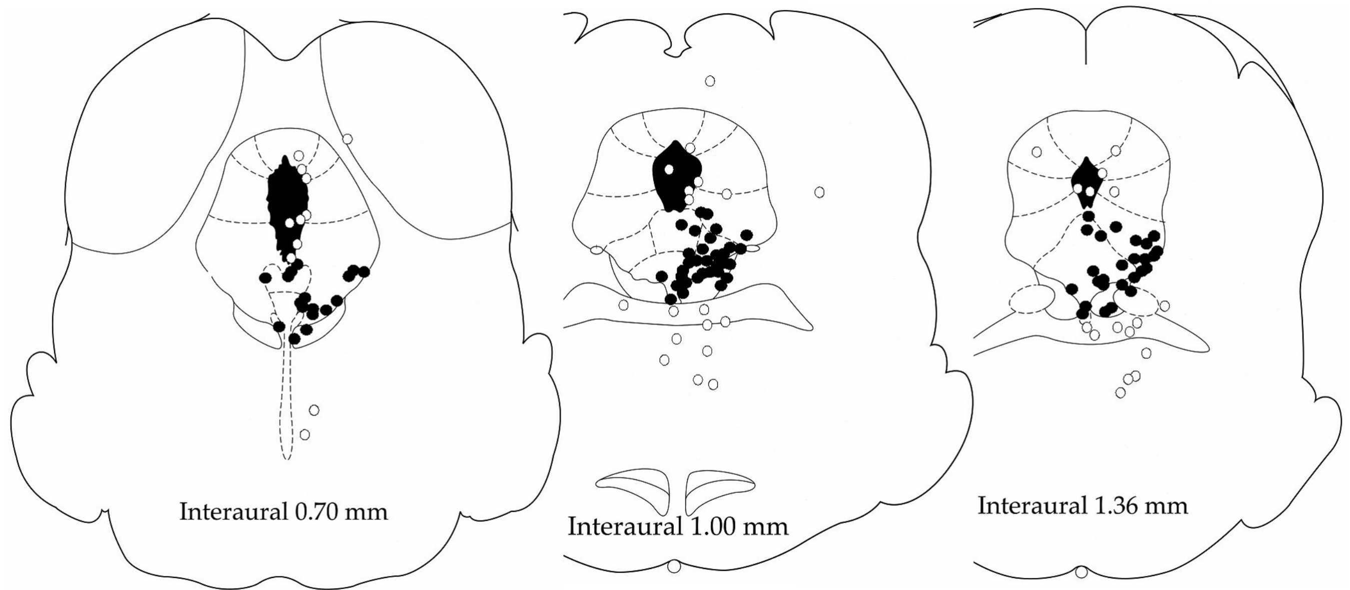

Figure 1.
Coronal sections of the midbrain showing the location of the microinjection sites. All injections were located in or along the border of the ventrolateral PAG (closed circles, N = 98). Injections outside this region were used as negative controls (open circles, N = 45). There was no difference in the location of the microinjections for the groups in any of the experiments. PAG images are taken from the Atlas of Paxinos and Watson (2005).

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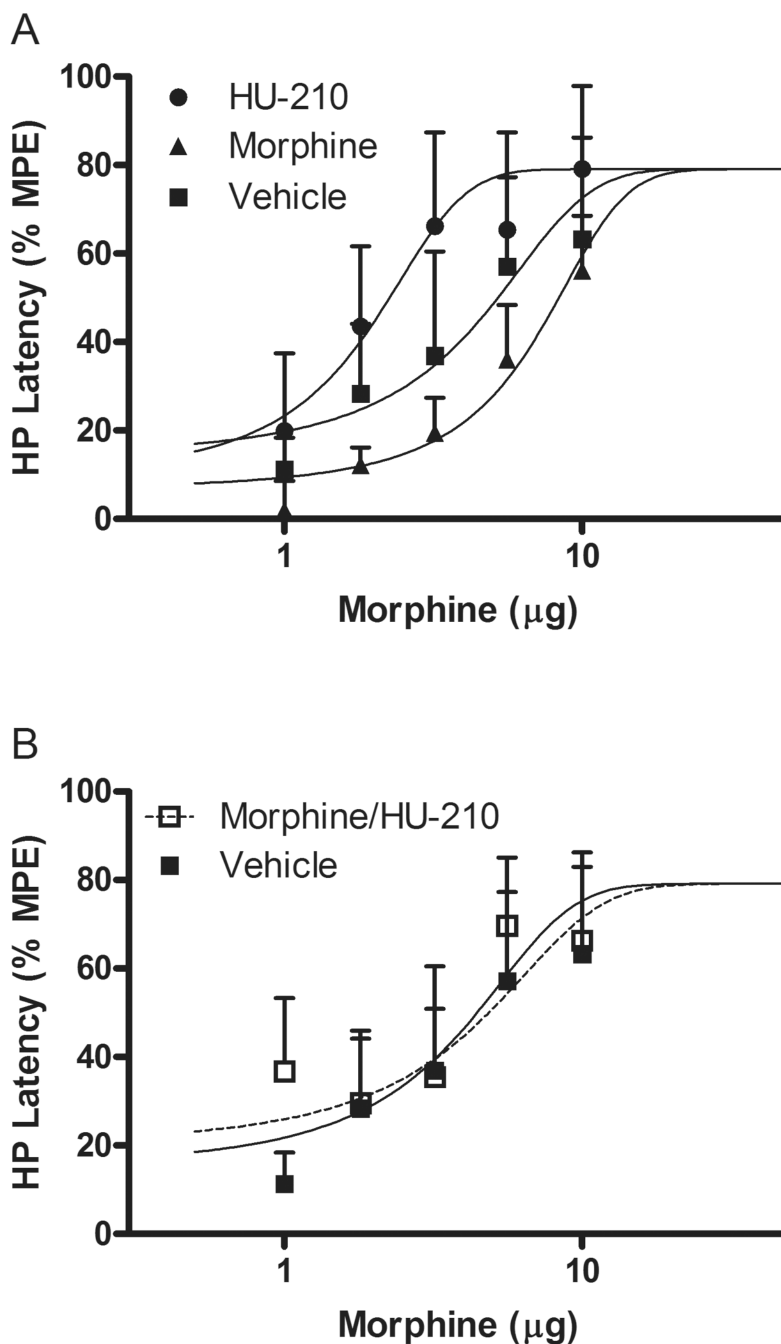

Figure 2.

Morphine antinociceptive potency following pretreatment with HU-210 and/or morphine. A: Repeated microinjection of morphine caused a rightward shift in the dose response curve for morphine antinociception as expected with the development of tolerance. Morphine potency was greatest in rats pretreated with HU-210 on Trials 1–4 as shown by the leftward shift in the morphine dose-response curve. B: Co-administration of morphine and HU-210 on Trials 1–4 attenuated the development of tolerance when morphine was microinjected on Day 3. The dose response curve for the vehicle group has been shown in both panel A and B to allow for visual comparison of shifts relative to vehicle. Average baseline hot plate latencies were 13.9 (morphine), 14.5 (HU-210), 13.5 (HU-210/morphine) and 13.9 (vehicle). See Table 1 for

D_{50} values. Fitted lines visually indicate trends and cannot be considered perfect fits to the data.

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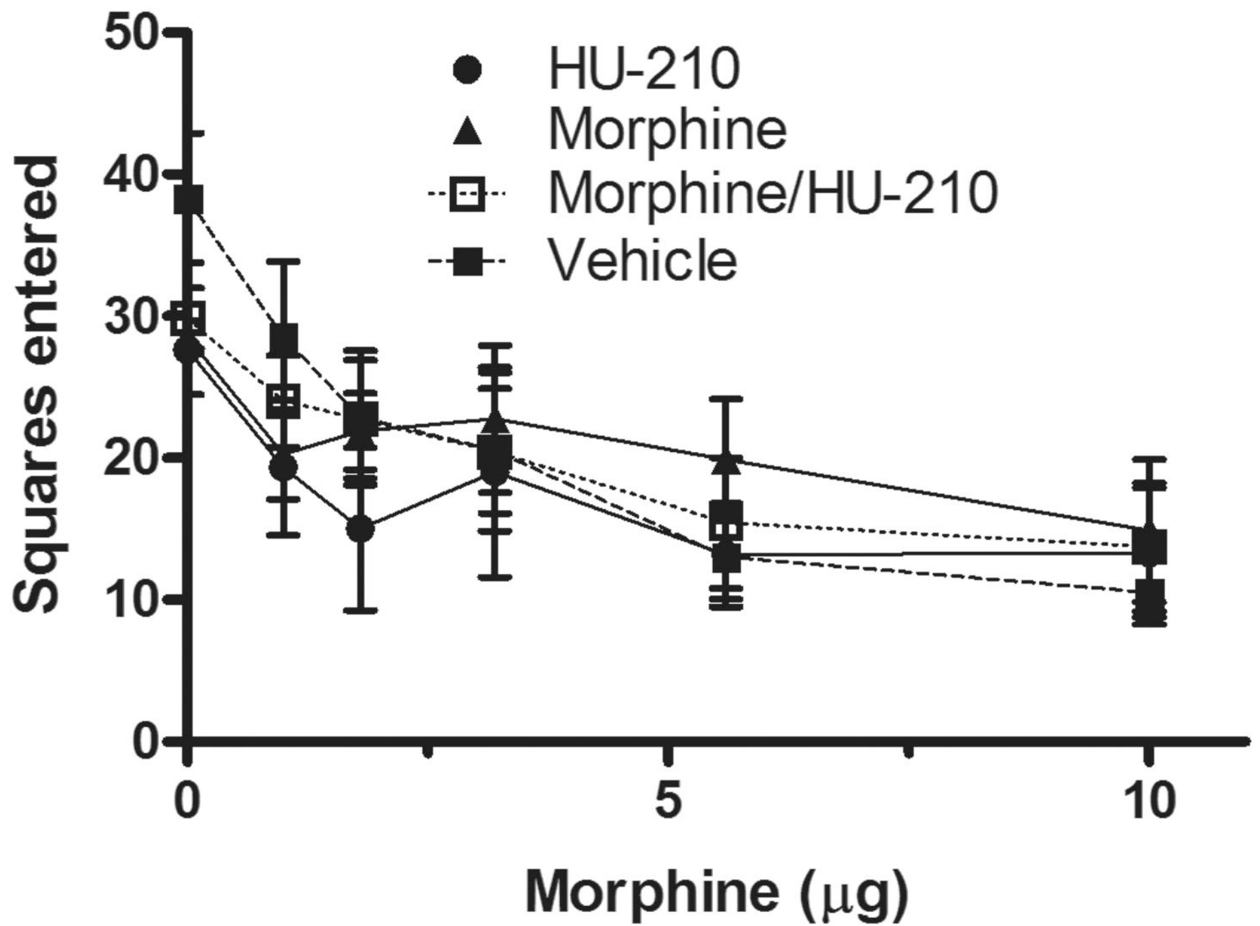

Figure 3.
Locomotor activity following morphine microinjections into the ventrolateral PAG on Trial 5. Morphine caused a dose-dependent decrease in locomotion for all groups. There was no significant difference in locomotion between any of the groups at any single dose.

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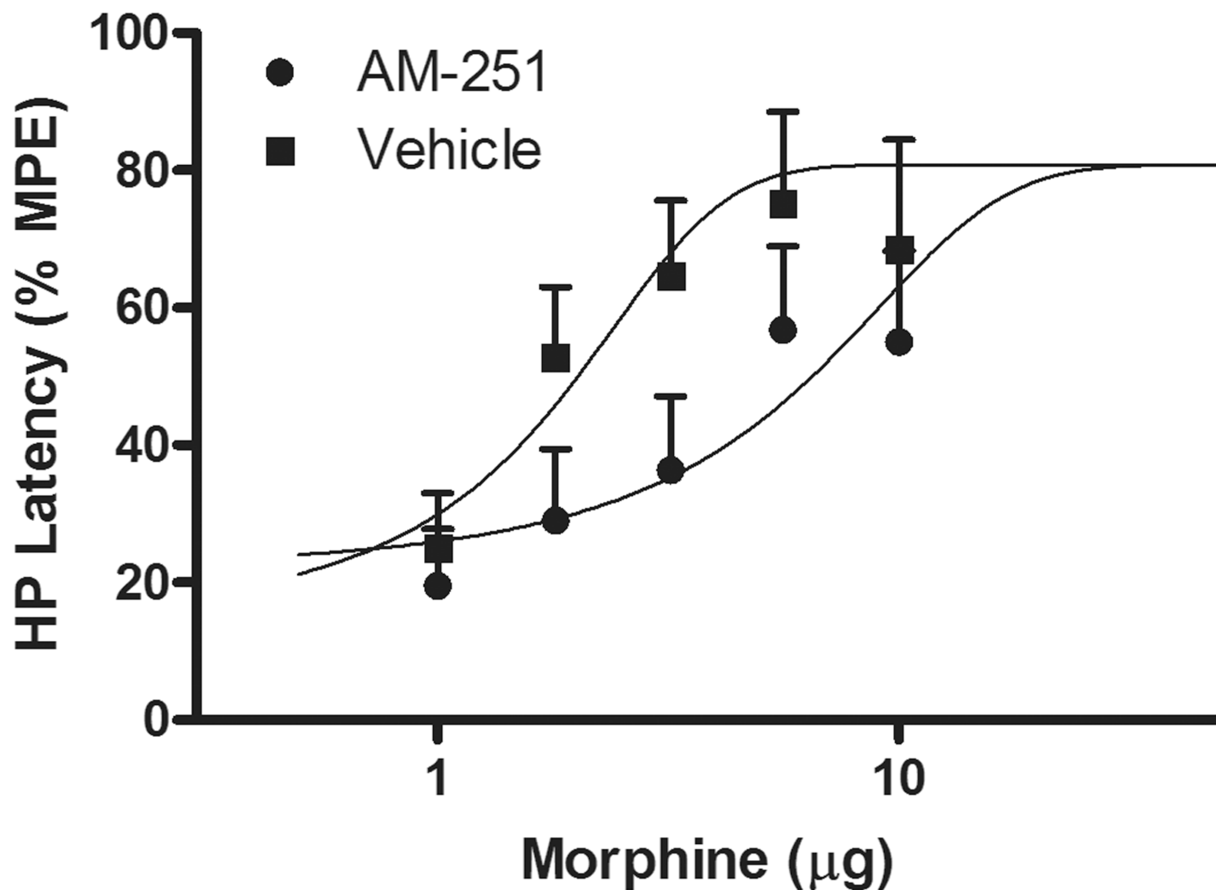

Figure 4.
Morphine's antinociceptive potency measured 2 days following pretreatment with i.p. 60% DMSO vehicle/intracranial HU-210 (squares) or i.p. AM-251/intracranial HU-210 (circles). Morphine potency was reduced in animals receiving the cannabinoid antagonist AM-251 in combination with HU-210, as shown by the rightward shift in the dose-response curve.

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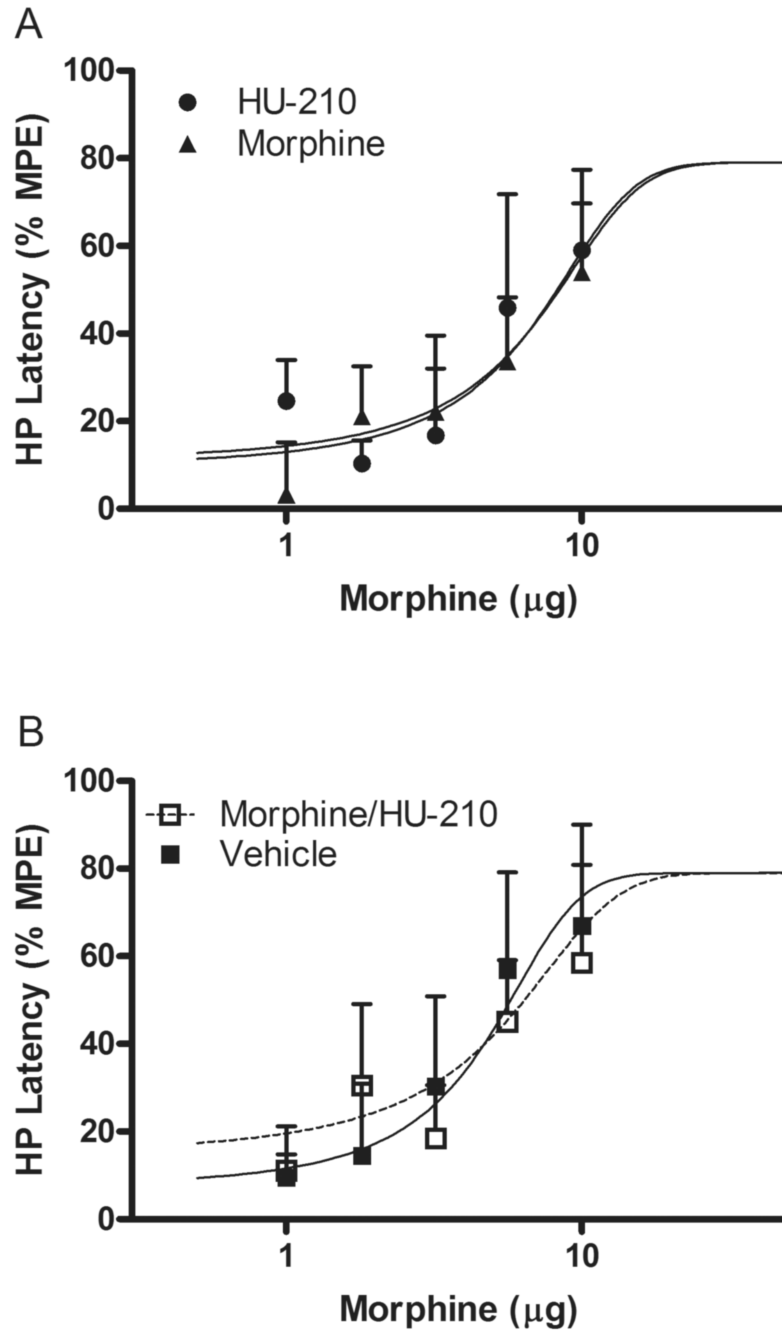

Figure 5.

Morphine potency measured 6 days following pretreatment with HU-210 and/or morphine microinjection into the ventrolateral PAG. Co-administration of morphine and HU-210 during Trials 1–4 failed to attenuate the development of tolerance when morphine was microinjected on Day 8. Fitted lines visually indicate trends and cannot be considered perfect fits to the data.

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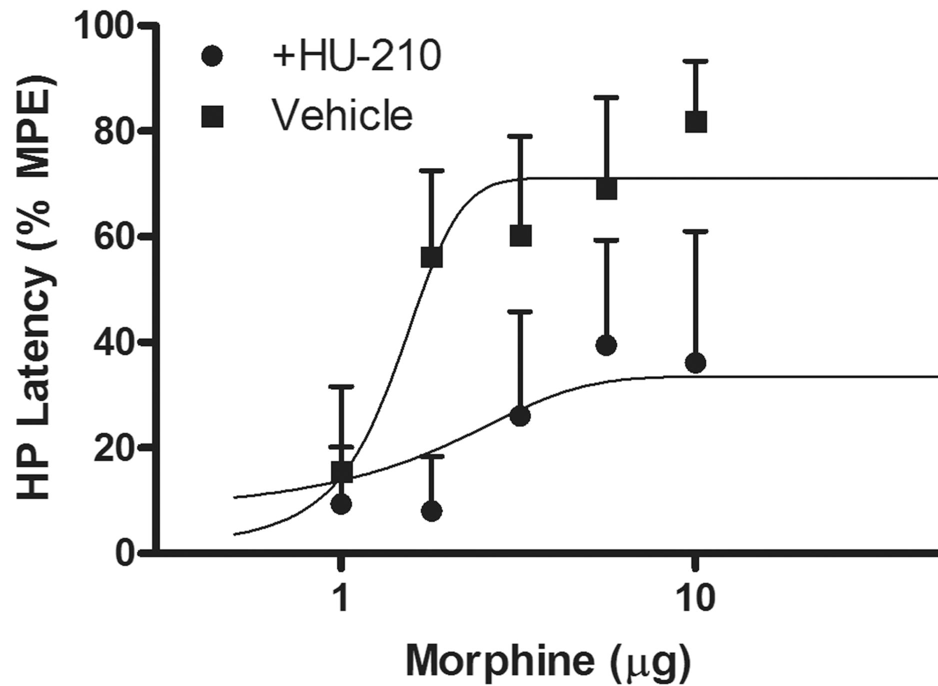

Figure 6.

Acute antinociceptive potency of morphine. Circles: first microinjection, morphine combined with 5μg HU-210, 4 subsequent microinjections of morphine (D_{50} = 2.58, 95% C.I. = − 0.56 – 5.73). Squares: 5 cumulative microinjections of morphine (D_{50} = 1.39, 95% C.I. = 0.75 – 2.03). An initial microinjection of HU-210 with morphine reduced the efficacy of morphine compared to animals microinjected solely with morphine.

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Table1

Comparison of morphine D_{50} values on Trial 5

Pretreatment	Day 3		Day 8	
	D_{50}	95% CI[a]	D_{50}	95% CI[a]
HU-210	1.77	0.55 – 2.99	5.82	2.11 – 9.53
Morphine/HU	2.54	0.05 – 5.11	5.61	2.27 – 8.95
Morphine	6.78	4.88 – 8.69	6.93	3.85 – 10.0
Vehicle	3.82	1.03 – 6.61	4.18	1.80 – 6.56

[a]confidence interval