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# Repeated cannabinoid injections into the rat periaqueductal gray enhance subsequent morphine antinociception

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ABSTRACT

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 Sprague–Dawley rats were injected twice a day for two days with *vehicle* (0.4 μL), morphine (5 μg/0.4 μL), HU-210 (5 μg/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.


## 1. 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 gray (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 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, 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

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morphine potency following repeated microinjections of the cannabinoid receptor agonist HU-210.

## 2. Methods

### 2.1. 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 (0.573 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.

### 2.2. 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 (0.226 mm), 11 mm long injection cannula inserted into and extending 2 mm beyond the tip of the 9 mm guide cannula. The 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.

### 2.3. 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 0.4 μL. In Experiments 2, 3, and 4, animals were given four injections of 0.4 μL during pretreatment, and five subsequent 0.4 μL 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 8.0 μg, resulting in cumulative quarter log doses of 3.2, 5.6, 10.0, and 18 μg. For the morphine cumulative dosing procedure, actual doses injected were 1.0, 0.8, 1.4, 2.4, and 4.4 μg, resulting in cumulative quarter log doses of 1.0, 1.8, 3.2, 5.6, and 10 μg.

### 2.4. 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 9–14 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.

### 2.5. 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, and 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.

### 2.6. 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 (09:30 and 16:00) 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 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.

### 2.7. 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 15 min later. This procedure was repeated twice a day for two days (Trials 1–4). Nociception was assessed using the hot plate test 30 min 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.

### 2.8. 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 was conducted on Days 3–7.

### 2.9. 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 ($N = 8$/group) 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 min apart and nociception and open field activity were assessed 15 min after each injection.

### 2.10. 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).

### 2.11. 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 0.05.

## 3. Results

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