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Cross-sensitization and cross-tolerance between exogenous cannabinoid antinociception and endocannabinoid-mediated stress-induced analgesia

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Summary

Footshock stress induces both endocannabinoid mobilization and antinociception. The present studies investigated behavioral plasticity in cannabinoid antinociceptive mechanisms following repeated activation using the tail-flick test. A secondary objective was to ascertain whether blockade of stress antinociception by the CB₁ antagonist rimonabant could be attributed to changes in locomotor activity. The cannabinoid agonist WIN55,212-2 induced hypoactivity in the open field relative to vehicle-treated controls. By contrast, rimonabant, administered at a dose that virtually eliminated endocannabinoid-mediated stress antinociception, failed to alter locomotor behavior (i.e. time resting, ambulatory counts, distance traveled) in rats subjected to the same stressor. Rats exposed acutely to footshock were hypersensitive to the antinociceptive effects of WIN55,212-2 and Δ^9 -tetrahydrocannabinol (Δ^9 -THC). The converse was also true; acute Δ^9 -THC and WIN55,212-2 administration potentiated stress antinociception, suggesting a bidirectional sensitization between endocannabinoid-mediated stress antinociception and exogenous cannabinoid antinociception. Stress antinociception was also attenuated following chronic relative to acute treatment with WIN55,212-2 or Δ^9 -THC. Repeated exposure to footshock (3 min/day for 15 days), however, failed to attenuate antinociception induced by either footshock stress or WIN55,212-2. Our results demonstrate that endocannabinoid-mediated stress antinociception cannot be attributed to motor suppression. Our results further identify a functional plasticity of the cannabinoid system in response to repeated activation. The existence of cross-sensitization between endocannabinoid-mediated stress antinociception and exogenous cannabinoid antinociception suggests that these phenomena are mediated by a common mechanism. The observation of stress-induced hypersensitivity to effects of exogenous cannabinoids may have clinical implications for understanding marijuana abuse liability in humans.

Keywords

cannabinoid; sensitization; stress antinociception; rimonabant; tolerance; footshock; anxiety; THC; endocannabinoid; sensitization; stress antinociception; rimonabant; tolerance; footshock; anxiety

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1. Introduction

Stress antinociception is a behavioral phenomenon in which animals are less responsive to noxious stimulation following exposure to an environmental stressor. Different parameters and durations of stress activate either opioid-dependent or opioid-independent analgesic mechanisms (Lewis et al., 1980; Terman et al., 1996). Previous work from our laboratories demonstrated that the coordinated release of the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide mediates opioid-independent stress antinociception by engaging cannabinoid CB₁ receptors (Hohmann et al., 2005; Suplita II et al., 2005, 2006). This discovery is consistent with the hypothesis that endocannabinoids, released under physiological conditions, produce adaptive changes in pain responses. However, the functional significance of the endocannabinoid signaling system to behavior remains incompletely understood.

Exogenous cannabinoids induce motor deficits (e.g. immobility, catalepsy) that may confound interpretation of behavioral studies of antinociception that largely measure motor responses to noxious stimulation (Martin et al., 1991). Electrophysiological studies demonstrate that analgesic effects of exogenous cannabinoids are independent of motor deficits induced by these compounds (Martin et al. 1996; Meng et al. 1998). Nonetheless, due to the potential for such confounds, it is necessary to demonstrate that apparent antinociceptive effects observed in behavioral studies are not experimental artifacts attributable to motor suppression. Here we examine the effects of the stressor used in our previous studies to induce stress antinociception (Connell et al., 2006; Hohmann et al., 2005; Suplita II et al., 2005, 2006) on the ambulatory behavior of rats. These studies demonstrate that the ability of rimonabant to attenuate stress antinociception cannot be attributed to changes in basal locomotor activity.

Tolerance and dependence develop in laboratory animals as well as humans following chronic exposure to synthetic cannabinoids (for review see Lichtman and Martin, 2005). Repeated once-daily exposure to intermittent footshock stress for two weeks results in tolerance to an opioid-dependent, but not an opioid-independent, form of stress antinociception (Lewis et al., 1981; Terman et al., 1986). Similarly, we showed that chronic treatment with the cannabinoid agonist WIN55,212-2 attenuated endocannabinoid-mediated stress antinociception (Hohmann et al., 2005). The present studies were conducted to further examine the functional plasticity of the endocannabinoid system in response to repeated activation.

We tested the hypothesis that a cross-sensitization and cross-tolerance would be observed between endogenous and exogenous cannabinoid antinociception. First, we examined the impact of exposure to footshock stress (using parameters known to induce endocannabinoid-mediated stress-induced analgesia) on antinociception induced by exogenous cannabinoids. Second, we evaluated the reverse contingency to determine if sensitization and tolerance between stress-induced and pharmacologically-induced antinociception was bidirectional. Third, we examined the impact of acute and chronic exposure to exogenous cannabinoids on endocannabinoid-mediated stress antinociception. Finally, we used repeated exposure to footshock stress to determine whether repetitive activation of the endocannabinoid system would induce tolerance to endogenous and exogenous cannabinoid antinociception. Preliminary results have been reported (Hohmann et al., 2005).

2. Methods

2.1 Animals

Two hundred and seven adult male Sprague-Dawley rats (275–350 g; Harlan, Indianapolis, IN) were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983). Rats were

individually housed upon arrival at the animal facility and thus were not tested in the presence of known cagemates (Langford et al., 2006). All efforts were made to minimize the number of animals used and their suffering.

2.2 Drugs

The CB₁ antagonist/inverse agonist SR141716A (rimonabant) and the CB₂ antagonist SR144528 were gifts from NIDA. Δ^9 -THC, naltrexone, morphine sulfate and WIN55,212-2 were purchased from Sigma-Aldrich (St. Louis, MO). Drugs were dissolved in emulphor:ethanol:saline (1:1:8 or 1:1:0) vehicle solution and administered via intraperitoneal (i.p.) injection in a volume of 1 ml/kg body weight. Morphine sulfate was dissolved in the same vehicle (1:1:8 emulphor:ethanol:saline) and administered subcutaneously (1 ml/kg bodyweight s.c.).

2.3 Behavioral testing

Stress antinociception was induced by exposure to continuous footshock (0.9 mA, AC current for 3 min (Lewis et al., 1980)), as described in our previously published work (Hohmann et al., 2005; Suplita II et al., 2005, 2006). Stress antinociception was quantified using the tail-flick test (D'Amour and Smith, 1941). The latency for rats to withdraw their tails from a radiant heat source was quantified before and after pharmacological manipulations and before and after exposure to footshock or no shock treatment. Animals were allowed to habituate to restraining tubes prior to assessment of tail-flick latencies. Withdrawal latencies to thermal stimulation of the tail were measured at 2-min intervals before and after footshock and before and after pharmacological manipulations. To assess stress antinociception, tail-flick latencies were calculated for each subject in 2-trial blocks. The ceiling latency was 10 sec in all studies, except where noted.

Experiment 1: Evaluation of the receptor mechanism underlying non-opioid stress - induced analgesia—This experiment was designed to test the hypothesis that non-opioid stress-induced analgesia was mediated by a cannabinoid CB₁ mechanism (see Hohmann et al., 2005). Rats received either the CB₁ antagonist rimonabant (5 mg/kg i.p.), the CB₂ antagonist SR144528 (5 mg/kg i.p.), the opiate antagonist naltrexone (14 mg/kg i.p.) or vehicle following determination of baseline tail-flick latencies. Twenty-five minutes following injection, rats were exposed to the footshock stressor (0.9 mA AC current, 3 min). Post-shock tail-flick latencies were monitored over 60 min. To evaluate the effects of rimonabant on basal nociceptive thresholds, separate groups received either rimonabant (5 mg/kg i.p.) or vehicle but were not exposed to the stressor. Tail-flick latencies were measured before injections (baseline) and over the same interval used to assess stress antinociception.

Experiment 2: Effects of rimonabant and footshock stress on locomotor activity—This experiment was designed to test the alternative hypothesis that endocannabinoid-mediated stress antinociception could be attributable to footshock-induced changes in locomotor activity that were blocked by rimonabant. Groups received either rimonabant (5 mg/kg i.p.) or emulphor: ethanol: saline (1:1:8) vehicle 30 min prior to exposure to the footshock stressor (3 min 0.9 mA) used to elicit stress antinociception. Separate groups received either WIN55,212-2 (5 mg/kg i.p.) or vehicle but were not subjected to footshock. All rats were placed in an automated open field arena (Med Associates, St. Albans, VT) 30 min following pharmacological manipulations and were free to explore the arena for fifteen minutes. During this time, behavior was automatically recorded by computerized analysis of photobeam interrupts (Med Associates). The Plexiglas arena was 43.2 × 3.2 × 30.5 cm and had a Plexiglas floor. Total time resting, ambulatory counts, and total distance traveled were monitored and recorded automatically. On day 1, baseline locomotor measurements were assessed in all rats. On day 2, rats were again placed in the open field arena 30 min following drug or vehicle

administration (i.e. 24 h following baseline assessments of locomotor activity). The same behaviors were monitored and quantified (in a 15 min interval) on both day 1 and day 2. Animals were removed from the arena 15 min following introduction into the open field. The interval evaluated corresponded to the maximal change in stress antinociception induced by footshock stress.

Experiment 3: Evaluation of cross-sensitization between endocannabinoid-mediated stress-induced analgesia and exogenous cannabinoid antinociception

—This experiment was designed to test the hypothesis that prior activation of the endocannabinoid system by footshock stress would induce behavioral sensitization to the antinociceptive effects of synthetic cannabinoids. Experiment 3 treatments are summarized in Table 1. Tail-flick latencies were initially measured before and after exposure to footshock stress on day 1. Δ^9 -THC or WIN55,212-2 was administered 24 h following exposure to footshock or no shock treatment. Animals were exposed to their home cages for the same times as the control (no shock) condition. On day 2, when rats no longer exhibited stress antinociception, tail-flick latencies were measured at 2-min intervals before and after pharmacological manipulations. Cannabinoid antinociception was quantified 28, 30, and 32 min post-injection for each rat to obtain a mean tail-flick response. This assessment interval corresponded to the maximal antinociceptive effect of the cannabinoid following i.p. injection.

General Methods for Tolerance Induction—Effects of chronic administration of Δ^9 -THC, WIN55,212-2 and morphine sulfate on endocannabinoid-mediated stress antinociception was assessed in separate studies (Experiments 4–6). These studies were designed to test the hypothesis that endocannabinoid-mediated stress-induced analgesia would be attenuated in rats rendered tolerant to cannabinoids but not to morphine. We also hypothesized that acute administration of cannabinoids would induce behavioral sensitization to the antinociceptive effects of stress. Injections occurred at the same time daily ($\pm$ 30 min) for all experiments. Stress antinociception was assessed when injection paradigms resulted in maximal antinociceptive tolerance or employed parameters validated previously in the literature (Oviedo et al., 1993; Terman et al., 1996).

Experiment 4: Effects of acute and chronic administration of the cannabinoid agonist Δ^9 -THC on endocannabinoid-mediated stress-induced analgesia

Experiment 4 treatments are summarized in Table 2. Tolerance to Δ^9 -THC (10 mg/kg i.p.) was induced using methods described previously (Oviedo et al., 1993). Rats received repeated once daily injections of either Δ^9 -THC (10 mg/kg i.p.) or vehicle over 14 days. A third group received a single injection of Δ^9 -THC (10 mg/kg i.p.) on day 14 only. In this study, all rats were subjected to footshock (0.9 mA AC current for 3 min) thirty-minutes following the last injection (day 14). Tail-flick latencies were measured on the test day before the last injection (baseline) and over 60 min following footshock. A short (30 min) delay between injection and assessment of stress antinociception was used to mimic the conditions employed by Oviedo et al. (1993). This protocol has been previously shown to produce a downregulation of cannabinoid receptors and induce tolerance to the locomotor effects of Δ^9 -THC (Oviedo et al., 1993).

Experiment 5a: Effects of acute and chronic administration of the cannabinoid agonist WIN55,212-2 on endocannabinoid-mediated stress-induced analgesia

—Experiment 5a treatments are summarized in Table 3a. Rats were rendered tolerant to WIN55,212-2-induced antinociception by administering once daily injections of WIN55,212-2 (10 mg/kg i.p.) or vehicle over 14 days as described previously (Hohmann et al., 2005). A third group (acute WIN55,212-2) received a single i.p. injection on day 14 only. The development of tolerance to WIN55,212-2-induced antinociception was verified prior to administration of the stressor by monitoring tail-flick latencies before and after injections performed on days 1,

7 and 14. Rats were exposed to the footshock stressor (0.9 mA AC current, 3 min) 24 h following the last injection of drug or vehicle (day 15). Tail-flick latencies were quantified before and after exposure to footshock. Stress antinociception was monitored over 60 min. The cut-off latency was 14 sec to permit detection of increases (i.e. sensitization) in stress antinociception following acute drug administration.

Experiment 5b: Effects of tolerance to cannabinoid antinociception on morphine antinociception—This experiment was designed to verify that chronic cannabinoid treatment did not suppress endocannabinoid-mediated stress-induced analgesia indirectly by inducing regulatory changes in mu-opioid responsive systems. Experiment 5b treatments are summarized in Table 3b. We examined the antinociceptive effects of morphine (2.5 mg/kg s.c. on day 15) in rats treated chronically with either WIN55,212-2 or vehicle in lieu of exposure to the stressor. This study employed the same cannabinoid tolerance induction regimen as described in Experiment 5a above. A submaximal dose of morphine was used so that it would be possible to observe enhancements as well as deficits in morphine-antinociception in cannabinoid-tolerant animals. After establishing stable baseline responding to thermal stimulation of the tail, morphine was administered to chronic WIN55,212-2 (10 mg/kg i.p. × 14 days) and chronic vehicle-treated groups. Morphine antinociception was subsequently assessed three times at 28, 30 and 32 min post injection; tail-flick latencies were averaged for each rat. This interval corresponded to the maximal antinociceptive effect of morphine observed following s.c. administration in pilot studies.

Experiment 6: Effects of tolerance to the opiate analgesic morphine on endocannabinoid-mediated stress-induced analgesia—This experiment was designed to test the hypothesis that endocannabinoid-mediated stress-induced analgesia is independent of mu opioid analgesia. Experiment 6 treatments are summarized in Table 4. Rats were rendered tolerant to morphine antinociception by once-daily injections of morphine sulfate (10 mg/kg s.c.) or vehicle over 7 days. This same injection paradigm was previously shown to attenuate opioid-mediated stress antinociception (Terman et al. 1986). Tolerance to morphine antinociception was verified prior to administration of the footshock stressor by monitoring tail-flick latencies before and after injections performed on days 1, 2 and 7. Approximately 24 h following the last injection (day 8), rats were exposed to the footshock stressor. The basal nociceptive threshold was assessed in the tail-flick test immediately prior to administration of the stressor. Stress antinociception was monitored over 60 min.

Experiment 7: Effect of repeated exposure to footshock stress on endocannabinoid-mediated stress-induced analgesia and exogenous cannabinoid antinociception—This experiment was designed to test the hypothesis that repeated exposure to the footshock stressor would induce tolerance to endogenous and exogenous cannabinoid antinociception. Experiment 7 treatments are summarized in Table 5. Separate groups of rats were exposed once per day for 15 days to the footshock stressor used to induce endocannabinoid-mediated stress antinociception (3 min, 0.9 mA AC current). A separate group of rats was similarly transported from the colony room to the testing room once per day for 15 days, but was not exposed to footshock stress (no shock control). Repeated exposure to footshock or no shock stress occurred at the same time daily ($\pm$ 30 min) for all experiments. Tail-flick latencies were measured three times at 2 min intervals before (baseline) and after the first and last exposure to the footshock stressor and before and after pharmacological manipulations. All groups received WIN55,212-2 (5 mg/kg i.p.) on day 16, approximately 24 h following the last exposure to footshock or no shock. Cannabinoid antinociception was subsequently assessed at 28, 30 and 32 min post-injection; tail-flick latencies were averaged for each rat. This interval corresponded to the maximal antinociceptive effect of WIN55,212-2 (i.p.) in pilot studies.

2.4 Statistical analyses

Data were analyzed using repeated-measures analysis of variance (ANOVA), one-way ANOVA, analysis of covariance (ANCOVA), and independent-samples or repeated-measures *t* tests, as appropriate. Planned comparisons were made using independent samples *t* tests. Post-hoc comparisons were performed using Fisher's PLSD tests to correct for inflated alpha error. $P < 0.05$ was considered significant.

3. Results

3.1 Experiment 1: The CB₁ antagonist rimonabant suppresses nonopioid stress-induced analgesia

Prior to exposure to the stressor, baseline tail-flick latencies did not differ between groups (Figure 1a). Brief, continuous footshock induced robust antinociception relative to baseline levels [$F(9,261) = 40.662$, $P < 0.0002$] (Figure 1a). The CB₁ antagonist rimonabant (5 mg/kg i.p.) markedly suppressed stress antinociception [$F(3,29) = 5.986$, $P < 0.003$] relative to all other groups. By contrast, neither the opiate antagonist naltrexone (14 mg/kg i.p.) nor the CB₂ antagonist SR144528 (5 mg/kg i.p.) altered stress antinociception over the same time course, as reported previously (Hohmann et al., 2005). These effects cannot be attributed to changes in basal nociceptive thresholds; rimonabant did not alter tail-flick latencies in the absence of the stressor (Figure 1b).

3.2 Experiment 2: Rimonabant-induced blockade of stress antinociception is independent of changes in locomotor activity

In the absence of the stressor, WIN55, 212-2 produced a characteristic suppression of locomotor activity relative to vehicle treatment. This hypoactivity was manifest as an increase in total time resting [$t(10) = 7.734$, $P < 0.0001$] and decreases in ambulatory counts [$t(10) = 2.76$, $P < 0.05$] and total distance traveled [$t(10) = 2.99$, $P < 0.05$] (Figure 1 c,e,g). By contrast, rimonabant, administered at a dose that virtually eliminated stress antinociception (see Figure 1a), failed to alter any parameter of post-shock locomotor activity relative to vehicle over the same interval [$P > 0.05$ for all comparisons] (Figure 1d,f,h). Locomotor behavior was also similar between groups prior to pharmacological manipulations (day 1) and prior to footshock or no shock treatment [$P > 0.05$ for all comparisons; data not shown].

3.3 Experiment 3: Footshock stress enhances the antinociceptive effects of WIN55,212-2 and Δ^9 -THC

Exposure to footshock stress elevated tail-flick latencies on day 1 prior to pharmacological manipulations [$t(14) = 9.14$, $P < 0.0001$ in Figure 2a; $t(21) = 17.96$, $P < 0.0002$ in Figure 2c]. Prior exposure to footshock stress enhanced the antinociceptive effects of both WIN55,212-2 (2.5 mg/kg, i.p.) [$t(14) = 9.597$, $P < 0.0001$; Figure 2b] and Δ^9 -THC (10 mg/kg, i.p.) [$t(14) = 9.597$, $P < 0.0001$; Figure 2d] on day 2. This enhancement was observed relative to non-shocked controls. By contrast, basal nociceptive thresholds did not differ between groups prior to stress or no stress treatment (day 1; Figure 2a,c) or prior to pharmacological manipulations (day 2; Figure 2b,d).

3.4 Experiment 4: Effects of acute and chronic Δ^9 -THC on stress antinociception

Acute treatment with Δ^9 -THC (10 mg/kg i.p.) induced a modest but reliable antinociceptive effect [$F(2,20) = 4.639$, $P = 0.022$] immediately prior to administration of the stressor (Figure 3). Acute Δ^9 -THC increased tail-flick latencies relative to groups receiving either vehicle or chronic Δ^9 -THC (10 mg/kg i.p. for 14 days). This observation demonstrates that chronic treatment with Δ^9 -THC produced tolerance to the antinociceptive effects of the cannabinoid. ANCOVA was subsequently used to analyze group differences in stress antinociception; tail-

flick latencies determined prior to stressor exposure were treated as the covariate. This same subanalgesic dose of Δ^9 -THC (10 mg/kg i.p.) markedly potentiated stress antinociception relative to groups treated chronically with Δ^9 -THC or vehicle [$F(2,19) = 5.447$, $P < 0.05$; $P < 0.05$ for each comparison] (Figure 3). Stress antinociception did not differ in groups treated chronically with Δ^9 -THC or vehicle (Figure 3).

3.5.1 Experiment 5a: Effects of acute and chronic WIN55,212-2 on stress antinociception

—Prior to administration of the stressor, repeated daily injections of WIN55,212-2 produced robust tolerance to the antinociceptive effects of the cannabinoid; tail-flick latencies were higher on day 1 compared to day 7 or day 14 of the repeated injection paradigm [$F(2,38) = 35.10$, $P < 0.0002$] (Figure 4a, inset). Acute treatment with WIN55,212-2 (10 mg/kg ip on day 14 only) also enhanced endocannabinoid-mediated stress antinociception relative to chronic treatment with WIN55,212-2 (10 mg/kg ip for 14 days) or vehicle [$F(2,26) = 28.50$, $P < 0.0002$] (Figure 4a). Stress antinociception was markedly attenuated in rats rendered tolerant to WIN55,212-2 relative to either vehicle or acute treatment with the agonist [$F(2,26) = 28.50$, $P < 0.0002$] (Figure 4a).

3.5.2 Experiment 5b: Morphine antinociception is not suppressed in cannabinoid-tolerant rats

—The attenuation of stress antinociception observed in cannabinoid-tolerant rats is unlikely to be attributed to regulatory changes in mu opioid tone. Rats rendered tolerant to WIN55,212-2 in the same injection paradigm showed no deficit in their antinociceptive response to morphine (2.5 mg/kg s.c.). At this dose, morphine reliably elevated tail-flick latencies ($F(1,11) = 7.91$, $P < 0.02$) in both groups (Mean tail-flick latency after morphine: 5.33 ± 0.21 s and 6.09 ± 0.19 s in control and cannabinoid tolerant animals). By contrast, the basal nociceptive threshold, assessed immediately prior to morphine administration did not differ between groups (mean tail-flick latency prior to morphine: control rats, 3.31 ± 0.06 s; cannabinoid tolerant rats, 3.50 ± 0.15 s; $P > .27$). Morphine antinociception was increased by $42.0 \pm 3.21\%$ and $37.6 \pm 2.18\%$ relative to baseline in cannabinoid tolerant and control rats, respectively. Moreover, morphine did not differentially alter tail-flick latencies relative to baseline in either cannabinoid tolerant or control rats. Furthermore, as noted previously (Hohmann et al., 2005), rats rendered tolerant to morphine displayed no deficits in endocannabinoid-mediated stress antinociception.

3.6 Experiment 6: Morphine tolerance does not alter endocannabinoid-mediated stress antinociception

Prior to administration of the stressor, chronic morphine treatment (10 mg/kg s.c. $\times$ 7 days) elicited a characteristic blunting of morphine antinociception; tail-flick latencies were higher in morphine-treated rats on day 2 of the repeated injection paradigm compared to day 7 [$t(14) = 11.03$, $P < 0.0002$] (Figure 4b, inset). Nonetheless, morphine tolerance failed to alter endocannabinoid-mediated stress antinociception (Figure 4b). Stress antinociception was similar in morphine-tolerant and control groups. Baseline tail-flick latencies, determined immediately prior to footshock, were also similar between the two groups.

3.7 Experiment 7: Endogenous and exogenous cannabinoid antinociception are preserved following repeated exposure to footshock stress

Repeated exposure to footshock stress (once daily exposure for 3 min for 15 days) did not alter endocannabinoid-mediated stress antinociception. Robust stress antinociception was observed following either one [$t(7) = 7.18$, $P = 0.002$, Welch's correction] or fifteen [$t(8) = 8.166$, $P < 0.0001$, Welch's correction] days of exposure to the stressor relative to non-shocked control groups (Figure 5a). The magnitude of stress antinociception was also similar after 1 or 15 days of repeated exposure to footshock (Figure 5a). Moreover, the antinociceptive effects of WIN55,212-2 (5 mg/kg i.p.) were not altered by repeated exposure to footshock. The

antinociceptive effect of WIN55,212-2 (on day 16) was similar in chronic shock and chronic non-shock groups. WIN55,212-2-induced antinociception was nonetheless increased relative to baseline in both groups [$F(1,28) = 13.32$, $P = 0.001$] (Figure 5b). Prior to cannabinoid administration, baseline tail-flick latencies were also similar between groups (Figure 5b). The basal nociceptive threshold, determined prior to each footshock exposure and prior to pharmacological manipulations, remained stable throughout the study in both groups (Figure 5a, b) ($P > 0.05$ for all comparisons).

4. Discussion

Our previous work demonstrated that opioid-independent stress antinociception is mediated by mobilization of endocannabinoids and subsequent activation of cannabinoid CB₁ receptors (Hohmann et al., 2005; Suplita II et al., 2005, 2006). In these studies, pharmacological blockade of CB₁ with rimonabant or the structurally similar CB₁ antagonist AM251 blocked the antinociceptive effects of stress. Here we provide evidence that stress antinociception (induced according to the same paradigm employed in our previous studies) is not an artifact of generalized motor suppression. As anticipated (Jarbe et al., 2002, 2003), systemic administration of the synthetic cannabinoid WIN55,212-2 produced hypoactivity in the open field. Blockade of CB₁ with rimonabant did not alter post-shock locomotor activity but, nonetheless, virtually eliminated endocannabinoid-mediated stress antinociception. Thus, stress antinociception cannot be attributed to freezing behavior or hypoactivity induced by exposure to footshock stress. Moreover, rimonabant blockade of footshock-induced antinociception cannot be attributed to changes in locomotor activity. The antinociceptive effects of synthetic cannabinoids correlate highly ($r = 0.96$) with suppressions of nociceptive neuronal activity (Martin et al., 1996). Importantly, synthetic cannabinoids directly modulate nociceptive neuronal activity within pain modulatory circuits in the rostral ventromedial medulla to induce analgesia independently of the motor effects of these compounds (Meng et al. 1998). Our results suggest that endocannabinoid mechanisms of stress antinociception, like exogenous cannabinoid antinociception, is independent of changes in motor activity.

The present data demonstrate a functional plasticity of the cannabinoid system in response to repeated activation. Repeated administration of cannabinoids can produce behavioral sensitization characterized by stereotyped activities associated with more harmful abused substances (Cadoni et al., 2001; Rubino et al., 2001). Here we present evidence for behavioral sensitization to the antinociceptive effects of synthetic cannabinoids following exposure to an acute environmental stressor. This behavioral sensitization was observed with multiple cannabinoid agonists (WIN55,212-2 and Δ^9 -THC) that bind to cannabinoid receptors and occurred in the absence of changes in basal nociceptive thresholds. Moreover, this sensitization was bidirectional; footshock stress enhanced the antinociceptive effects of exogenous cannabinoids and exogenous cannabinoids enhanced endocannabinoid-mediated stress antinociception. These latter effects were receptor-mediated; sensitization in stress antinociception was apparent following acute administration of cannabinoid agonists (WIN55,212-2 and Δ^9 -THC) but was attenuated following chronic administration of these compounds. A greater attenuation of stress antinociception was observed in WIN55,212-2-tolerant relative to Δ^9 -THC-tolerant rats. Differences in the injection paradigm and the degree of tolerance induced by the full (WIN55,212-2) and partial (Δ^9 -THC) cannabinoid agonists may account for these differences. The neurochemical specificity of this tolerance was also demonstrated herein; tolerance to cannabinoid antinociception attenuated endocannabinoid-mediated stress antinociception but did not suppress morphine antinociception. Moreover, rendering rats tolerant to morphine failed to suppress endocannabinoid-mediated stress antinociception. The bidirectional nature of the cross-sensitization observed between endocannabinoid-mediated stress antinociception and exogenous cannabinoid antinociception

suggests that a common mechanism underlies these two phenomena. More work is necessary to identify the biochemical mechanism underlying the observed sensitization.

Rats treated chronically with Δ^9 -THC (10 mg/kg i.p. per day for 14 days and sacrificed 30 min after the last injection) show homogenous downregulations in brain cannabinoid receptor binding (decrease in $B_{\max}$) that are attributable to a lowering of capacity (Oviedo et al., 1993). By contrast, the acute dose of Δ^9 -THC employed here, administered 30 min prior to sacrifice, increased cannabinoid receptor affinity (K_d) (Oviedo et al., 1993). Footshock-induced endocannabinoid mobilization could produce behavioral sensitization by increasing cannabinoid receptor affinity and/or by facilitating activation of signal transduction pathways. Systemically administered cannabinoids activate spinal as well as supraspinal antinociceptive mechanisms (Walker and Hohmann, 2005); these mechanisms could synergize with antinociceptive effects mediated by stress-induced endocannabinoid mobilization (Hohmann et al., 2005; Suplita II et al., 2006).

Our data raise the possibility that chronic exposure to exogenous cannabinoids may render the endocannabinoid system less responsive under physiological conditions. Considering the importance of this endogenous system in regulating neuronal excitability (Chevalleyre and Castillo, 2003; Monory et al., 2006) as well as nociception, learning, memory, and appetitive motivation (Freund et al., 2003; Hill et al., 2005; Hohmann et al., 2005; Lichtman et al., 2002; Rademacher and Hillard, 2007), disruptions in endocannabinoid transmission could have a far-reaching, adverse impact. For example, the endocannabinoid system regulates extinction of aversive memories (Marsicano et al., 2002) through a habituation-like process (Kamprath et al., 2006). Without intact endocannabinoid function, animals are unable to stop evincing fear-typical responses such as freezing in response to a tone paired previously with footshock (Marsicano et al., 2002). By contrast, enhancing the bioavailability of endocannabinoids by blocking their reuptake facilitates extinction (Chhatwal et al., 2005). Long-term alterations in endocannabinoid function could therefore impair the system's ability to participate in normal emotional habituation and extinction processes, thereby increasing an individual's vulnerability to major depression (Hill et al., 2005) and other disorders involving deficient stress adaptation mechanisms (Di Marzo and Petrosino, 2007; Viveros et al., 2005).

Acute effects of smoked marijuana on antinociception, hypothermia, and catalepsy result from activation of CB_1 by Δ^9 -THC (Varvel et al., 2005). By contrast, non- Δ^9 -THC constituents of marijuana influenced these pharmacological effects minimally, if at all (Varvel et al., 2005). Thus, the observed cross-sensitization between pharmacological effects of Δ^9 -THC and stress-induced activation of the endocannabinoid system may have clinical relevance to understanding marijuana use and abuse in humans. If, as our data indicate, stress can produce sensitization to exogenous cannabinoids like Δ^9 -THC, it is reasonable to postulate that physiological and psychological effects of acute cannabis intoxication could be potentiated in individuals experiencing adverse environmental stress. One result might be an increased likelihood of reuse or establishment of a self-medicating routine involving cannabis and/or other drugs of abuse (Butters, 2002; Newcomb and Harlow, 1986). Thus, it is noteworthy that CB_1 antagonists have found therapeutic applications in reducing recidivism in smokers (Cohen et al., 2005; Tucci et al., 2006) and other substance abusers (Maldonado et al., 2006).

Repeated once daily exposure to intermittent footshock stress (20 min at 0.2 Hz $\times$ 14 days) induces tolerance to opioid-mediated stress antinociception (Lewis et al., 1981; Terman et al., 1986). Thus, it is noteworthy that repeated daily exposure to brief, continuous footshock stress (3 min $\times$ 15 days) does not induce tolerance to opioid-mediated stress antinociception (Lewis et al., 1981; Terman et al., 1986) and produced no deficit in endocannabinoid-mediated stress antinociception in our study. Moreover, WIN55,212-2 produced similar antinociceptive effects in rats exposed repeatedly to the footshock stressor and rats not subjected to footshock. These

observations reinforce the view that endocannabinoid- and opioid-mediated environmentally-induced analgesic mechanisms are not functionally redundant. More work is necessary to determine whether endocannabinoids play a role in opioid-mediated stress antinociception such as that elicited by acute or repeated exposure to intermittent footshock. Repeated daily exposure to the present stressor was likely insufficient to produce a downregulation of cannabinoid receptors or regulatory changes in endocannabinoid mobilization. However, other stressors (Hill et al., 2005; Patel et al., 2004, 2005b) or challenges (Petrosino et al., 2007) which induce different patterns of endocannabinoid mobilization could be associated with different behavioral phenotypes.

Endocannabinoids are synthesized on demand in a stimulation-contingent fashion (Piomelli, 2003; Vaughan and Christie, 2005). The lack of antinociceptive tolerance observed following repeated activation of the endocannabinoid system with footshock stress may, therefore, have clinical implications. Therapeutic strategies which indirectly enhance endocannabinoid signaling may be less susceptible to tolerance than approaches relying on global CB₁ receptor activation via direct agonist administration. Endocannabinoid signaling may be enhanced by inhibiting enzymatic hydrolysis or by blocking reuptake (Cravatt and Lichtman, 2003; Piomelli, 2003, 2005; Hohmann et al., 2005; Suplita II et al., 2005; 2006). These latter strategies circumvent global CB₁ activation, thereby avoiding undesirable physiological and psychoactive effects associated with direct CB₁ activation. Our data suggest that therapeutic strategies that enhance levels of endocannabinoids may be useful for treating stress- and pain-related disorders.

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Fig. 1.

Stress antinociception is attenuated by a cannabinoid CB₁ mechanism independent of changes in locomotor activity. (a) The CB₁ antagonist rimonabant (5 mg/kg i.p.) attenuates stress antinociception in the tail-flick test. The opiate antagonist naltrexone (14 mg/kg i.p.) and the CB₂ antagonist SR144528 (5 mg/kg i.p.) failed to alter stress antinociception (Hohmann et al., 2005). (b) Rimonabant (5 mg/kg i.p.) does not alter basal nociceptive thresholds relative to vehicle in the absence of the stressor. The cannabinoid agonist WIN55,212-2 (5 mg/kg i.p.) (c) increases total time resting, and decreased (e) ambulatory behavior and (g) total distance traveled relative to vehicle in the absence of the stressor. (d,f,h) Rimonabant (5 mg/kg i.p.), administered at a dose that suppresses stress antinociception (panel a), did not alter locomotor

activity (total time resting, ambulatory counts, total distance traveled) relative to vehicle following exposure to the stressor. Mean $\pm$ S.E.M. $*P < 0.05$, $***P < .001$ versus vehicle control (t-test); $n = 6-11$ /group.

Fig. 2.

Footshock stress enhances the antinociceptive effects of cannabinoid agonists WIN55,212-2 and Δ^9 -tetrahydrocannabinol. (a, c) Footshock, but not nonshock, elicits antinociception in the tail-flick test prior to pharmacological manipulations. (b) The antinociceptive effects of WIN55,212-2 (2.5 mg/kg i.p.) and (d) Δ^9 -THC (10 mg/kg i.p.) were enhanced relative to control (no shock) conditions. Cannabinoids were administered 24 h following exposure to footshock stress (see Table 1). Mean $\pm$ S.E.M. * $P < 0.05$, *** $P < 0.001$ versus non-shocked controls (t-tests); $n = 8-12$ /group.

Fig. 3.

Acute administration of the cannabinoid agonist Δ^9 -THC (10 mg/kg i.p. on day 14 only) enhances stress antinociception relative to vehicle or chronic treatment with Δ^9 -THC (10 mg/kg i.p. daily for 14 days). Footshock was administered 30 min following the last injection (see Table 2). * $P < 0.05$, ** $P < 0.01$ versus other groups (ANCOVA, Fisher's PLSD test). ^x $P < 0.05$ versus chronic Δ^9 -THC group; $n = 7-8$ /group.

Fig. 4.

Endocannabinoid-mediated stress antinociception is attenuated in rats rendered tolerant to the cannabinoid agonist WIN55,212-2. (a) Repeated once-daily administration of WIN55,212-2 (10 mg/kg i.p. $\times$ 14 days) attenuates stress antinociception relative to vehicle or acute WIN55,212-2 (10 mg/kg i.p. on day 14 only) treatment. Acute treatment with WIN55,212-2 enhanced stress antinociception relative to vehicle or chronic WIN55,212-2 treatment. Inset: Chronic treatment with WIN55,212-2 induces tolerance to cannabinoid antinociception prior to administration of the stressor (see Table 3a). (b) Chronic morphine treatment (10 mg/kg s.c. $\times$ 7 days) does not attenuate endocannabinoid-mediated stress antinociception relative to controls treated with vehicle (s.c.) at the same times. Inset: Chronic treatment with morphine attenuates morphine antinociception prior to administration of the stressor. The stressor was administered 24 h following the last injection (see Table 4). * $P < 0.05$ versus other groups; + $P < 0.05$ versus vehicle; $\times P < 0.05$ versus acute WIN55,212-2 (ANOVA, t-test), $n = 8-11/\text{group}$.

Fig. 5. WIN55,212-2-induced antinociception is preserved following repeated exposure to footshock stress. (a) No deficit in footshock-induced stress antinociception was observed following 1 or 15 daily exposures to the stressor used to induce endocannabinoid-mediated stress antinociception (0.9 mA; 3 min). The increase in tail-flick latencies observed subsequent to footshock exposure on day 1 is similar to that observed following 15 days of repeated exposure to footshock. (b) The antinociceptive effects of WIN55,212-2 (5 mg/kg i.p., administered acutely on day 16 only) are similar in groups subjected to chronic shock (3 min continuous footshock $\times$ 15 days) or chronic non-shock at the same times (see Table 5). ** $P < 0.05$, *** $P < 0.001$ versus non-shocked rats (t-test); $n = 8/\text{group}$.

Table 1

Summary of Experiment 3 treatments. Stress antinociception was assessed on day 1. The same animals received Δ^9 -THC (10 mg/kg i.p.) or WIN55,212-2 (2.5 mg/kg i.p.) on day 2 to assess cannabinoid antinociception.

	Day	
	1	2
Baseline TF	+	–
Shock (S)/No shock (NS)	S/NS	–
Post-shock TF	+	–
Pre-injection TF	–	+
Acute THC (+)/WIN (++)	–	+ / ++
Post-injection TF	–	+

Between-subjects variables are designated by symbols in bold.

All other symbols indicate within-subjects variables.

Abbreviations: **S**, shock administered; **NS**, no shock administered; +, inject THC; ++, inject WIN; +, treatment present; –, treatment absent; TF, tail-flick assessment; THC, Δ^9 -tetrahydrocannabinol; WIN, WIN55,212-2

Schedule for Experiment 4. Animals received either once daily injections of Δ^9 -THC (10 mg/kg i.p. $\times$ 14 days) or vehicle or acute treatment with Δ^9 -THC (10 mg/kg i.p. on day 14). Stress antinociception was assessed on day 14, 30 min following the last injection.

Table 2

	Day													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Baseline TF	+	-	-	-	-	-	+	-	-	-	-	-	-	+
Chronic THC (+)/Veh (-)	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Acute THC (+)	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Post-injection TF	+	-	-	-	-	-	+	-	-	-	-	-	-	+
Pre-shock TF	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Shock	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Post-shock TF	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Between-subjects variables are designated by symbols in bold. All other symbols indicate within-subjects variables.

Abbreviations: +, inject THC; -, inject Veh; +, treatment present; -, treatment absent; TF, tail-flick assessment; THC, Δ^9 -tetrahydrocannabinol; Veh, vehicle

Table 3

Table 3a. Schedule for Experiment 5a. Animals received either once daily injections of WIN55,212-2 (10 mg/kg i.p. $\times$ 14 days) or vehicle or acute treatment with WIN55,212-2 (10 mg/kg i.p. on day 14). Stress antinociception was assessed on day 15 in all groups.

	Day														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Baseline TF	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Chronic WIN (+)/Veh (-)	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-
Acute WIN (+)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Post-injection TF	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Pre-shock TF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Shock	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Post-shock TF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 3b. Summary of Experiment 5b treatments. Animals received either once daily injections of WIN55,212-2 (10 mg/kg i.p. $\times$ 14 days) or vehicle. Morphine (2.5 mg/kg s.c.) antinociception was assessed on day 15 in all groups.

	Day														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Baseline TF	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Chronic WIN (+)/Veh (-)	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-
Post-injection TF	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Pre-morphine TF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Acute morphine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Post-morphine TF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Between-subjects variables are designated by symbols in bold. All other symbols indicate within-subjects variables.

Abbreviations: +, inject WIN; -, inject Veh; +, treatment present; -, treatment absent; TF, tail-flick assessment; WIN, WIN55,212-2; Veh, vehicle

Summary of Experiment 6 treatments. Animals received once daily injections of morphine (10 mg/kg s.c. $\times$ 7 days) or vehicle. Stress antinociception was assessed on day 8 in all groups.

Table 4

	Day							
	1	2	3	4	5	6	7	8
Baseline TF								
Morphine (+)/Veh (-)	+	+	-	-	-	-	+	-
Post-injection TF	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-
	+	+	-	-	-	-	+	-
Pre-shock TF	-	-	-	-	-	-	-	+
Shock	-	-	-	-	-	-	-	+
Post-shock TF	-	-	-	-	-	-	-	+

Between-subjects variables are designated by symbols in bold. All other symbols indicate within-subjects variables.
Abbreviations: +, inject morphine; -, inject Veh; +, treatment present; -, treatment absent; TF, tail-flick assessment; Veh, vehicle

Summary of Experiment 7 treatments. Animals were subjected to once daily exposure to footshock (3 min, 0.9 mA) or no shock over 15 days. Antinociception induced by WIN55,212-2 (5 mg/kg i.p.) was assessed in all groups on day 16.

Table 5

	Day															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Baseline TF																
Shock (S)/No shock (NS)	+															
Post-shock TF	+															
Pre-WIN TF																
Acute WIN																
Post-WIN TF																

Between-subjects variables are designated by symbols in bold. All other symbols indicate within-subjects variables.

Abbreviations: S, shock administered; NS, no shock administered; +, treatment present; -, treatment absent; TF, tail-flick assessment; WIN, WIN55,212-2