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Differential effects of endocannabinoid catabolic inhibitors on morphine withdrawal in mice

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Author Disclosures

Contributors

T.F. Gamage conducted the bulk of the studies, data analyses, and writing of the manuscript. B.M. Ignatowska-Jankowska conducted a subset of the experiments as well as contributed to the data analysis and manuscript writing. P. P. Muldoon participated in the experimental design of CPA experiments. B.F Cravatt contributed to the experimental design of the study. M.I Damaj provided guidance in all place preference experiments. A.H. Lichtman oversaw all aspects of the studies and contributed to the writing of the manuscript. All authors have read and approve the submitted version of the manuscript.

Conflict of Interest

None of the authors report a conflict of interest that could have influenced, or be perceived to influence, this work.

Abstract

Background—Inhibition of endocannabinoid catabolic enzymes fatty acid amide hydrolase (FAAH) and/or monoacylglycerol lipase (MAGL) reduces somatic morphine withdrawal signs, but its effects on aversive aspects of withdrawal are unknown. The present study investigated whether Δ^9 -tetrahydrocannabinol (THC), the MAGL inhibitor JZL184, the FAAH inhibitor PF-3845, or the dual FAAH/MAGL inhibitor SA-57 would reduce acquisition of morphine withdrawal-induced conditioned place avoidance (CPA) and jumping.

Methods—Mice were implanted with placebo or 75 mg morphine pellets, 48 h later injected with naloxone or saline and placed in the conditioning apparatus, and assessed for CPA at 72 h. Subjects were also observed for jumping behavior following naloxone challenge.

Results—Naloxone (0.056 mg/kg) produced robust CPA in morphine-pelleted, but not placebo-pelleted, mice. Morphine pretreatment prevented the occurrence of withdrawal CPA and withdrawal jumping, while clonidine (an α_2 adrenergic receptor agonist) only blocked withdrawal CPA. THC, JZL184, and SA-57 significantly reduced the percentage of mice that jumped during the conditioning session, but did not affect acquisition of withdrawal CPA. PF-3845 did not reduce morphine withdrawal CPA or jumping. Finally, neither THC nor the endocannabinoid catabolic enzyme inhibitors in non-dependent mice elicited a conditioned place preference or aversion.

Conclusions—These findings suggest that inhibiting endocannabinoid catabolic enzymes reduces somatic morphine withdrawal signs, but not aversive aspects as inferred in the CPA paradigm. The observation that non-dependent mice administered inhibitors of endocannabinoid degradation did not display place preferences is consistent with the idea that endocannabinoid catabolic enzymes might be targeted therapeutically, with reduced risk of abuse.

Keywords

opioid; morphine; dependence; withdrawal; cannabinoid; fatty acid amide hydrolase (FAAH); monoacylglycerol lipase; cannabinoid; THC; conditioned place aversion (CPA); conditioned place preference (CPP)

1. INTRODUCTION

Opioid abuse and dependence continue to present a serious threat to public health (Johnston et al., 2010). Fear of withdrawal symptoms that include diarrhea, emesis, body aches, anxiety, dysphoria (Farrell, 1994; Gossop, 1988; Jasinski, 1981; Wesson and Ling, 2003) are thought to contribute to the maintenance of drug-taking in opioid dependent individuals. Likewise, continued opioid use alleviates the withdrawal state, thus serving as a negative reinforcer (Koob and Le Moal, 2005). Current available treatments for opioid dependence, such as methadone and buprenorphine, possess their own abuse liability (Cicero and Inciardi, 2005) and are not fully effective at alleviating withdrawal (Dyer et al., 1999; Kuhlman et al., 1998). Thus, new pharmacotherapies that lack abuse potential are needed to alleviate opioid withdrawal.

Extracts from cannabis and the primary constituent of marijuana, Δ^9 -tetrahydrocannabinol (THC), have long been known to ameliorate somatic morphine withdrawal signs (Birch, 1889; Hine et al., 1975). THC produces the bulk of its pharmacological effects through two known G-protein coupled receptors, cannabinoid type-1 (CB₁; Matsuda et al., 1990) and type-2 (CB₂; Munro et al., 1993). These receptors, as well as the endogenous cannabinoids (endocannabinoids) 2-arachidonoylglycerol (2-AG; Mechoulam et al., 1995; Sugiura et al., 1995) and *N*-arachidonylethanolamine (anandamide, AEA; Devane et al., 1992) comprise the endogenous cannabinoid system. These endocannabinoids are rapidly degraded by the respective enzymes fatty acid amide hydrolase (FAAH; Cravatt et al., 2001) and monoacylglycerol lipase (MAGL; Dinh et al., 2002). Selective inhibitors of these endocannabinoid degradative enzymes reduce somatic signs of opioid withdrawal (e.g. jumping, paw fluttering, head/body shaking, weight loss, diarrhea; Ramesh et al., 2013, 2011). However, it is unknown whether the anti-withdrawal effects extend to the affective components of morphine withdrawal.

Opioid-dependent individuals undergoing withdrawal experience aversive subjective effects, a process that is modeled in the Pavlovian conditioned place avoidance (CPA) paradigm. In this assay morphine-dependent rats (Gracy et al., 2001; Hand et al., 1988; Parker and Rennie, 1992; Schnur et al., 1992; Stinus et al., 2000, 1990; Watanabe et al., 2003) or mice (Broseta et al., 2005; Maldonado et al., 2003; Olson et al., 2006; Sato et al., 2005; Shoblock and Maidment, 2005) undergo conditioning trials in which naloxone precipitates an aversive interoceptive stimulus that is paired with a distinct chamber. Following subsequent placement into the test apparatus, the subjects spend less time in the conditioning chamber than in the control chamber (i.e., CPA). In this assay, lower doses of naloxone produce CPA than those doses necessary to elicit somatic withdrawal signs (Caillé et al., 1999; Frenois et al., 2002). Furthermore, the α_2 adrenergic agonist clonidine, which is known to reduce opioid withdrawal in humans (Gold et al., 1978; Gossop, 1988), attenuates opioid withdrawal CPA (Kosten, 1994; Schulteis et al., 1998a). Given the colocalization of CB₁ and mu opioid receptors in the locus coeruleus (Scavone et al., 2010), periaqueductal grey (Wilson-Poe et al., 2012) and nucleus accumbens (Pickel et al., 2004), cannabinoid receptors are advantageously positioned to compensate for the hyperactivity in neurons that are key to the expression of both somatic and aversive aspects of opioid withdrawal (Frenois et al., 2002; Lane-Ladd et al., 1997; Nestler and Tallman, 1988; Stinus et al., 1990; Widnell et al., 1994).

The purpose of the present study was to test whether stimulation of CB₁ receptors via administration of THC or inhibition of endocannabinoid catabolic enzymes would prevent the acquisition of naloxone-precipitated morphine withdrawal CPA and withdrawal-related jumping behavior in mice. To this end, the MAGL inhibitor JZL184 (Long et al., 2009a) or FAAH inhibitor PF-3845 (Ahn et al., 2009) was administered at the time of conditioning. In addition, we evaluated whether the dual FAAH/MAGL inhibitor SA-57 (Niphakis et al., 2012) would prevent morphine withdrawal CPA. Of note, combined inhibition of these two major endocannabinoid hydrolytic enzymes elicits enhanced cannabimimetic activity compared with single enzyme inhibition (Long et al., 2009b; Seillier et al., 2014; Wise et al., 2012). Moreover, each of these inhibitors reduces naloxone-precipitated and spontaneous

somatic withdrawal signs in morphine-dependent mice (Ramesh et al., 2013, 2011). Finally, each inhibitor was tested in non-dependent mice to ascertain whether the compounds elicited reward-related or aversive effects in the place conditioning assay.

2. MATERIAL AND METHODS

2.1. Subjects

Male ICR mice (6–8 weeks old; Harlan, Indianapolis, IN) with a body mass of 27–32 g were used for all experiments. Mice were group-housed (four per cage) on a 12/12 light/dark cycle (lights on at 0600 h) and given food and water *ad libitum*. All animal protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee, were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 2011), and efforts were made to minimize animal suffering and number of animals used.

2.2. Drugs

Morphine (75 mg) pellets, placebo pellets, morphine sulfate, and THC were obtained from the National Institute on Drug Abuse (Rockville, MD). Naloxone HCl and clonidine HCl were purchased from Sigma Aldrich (St. Louis, MO). JZL184, PF-3845, and SA-57 were synthesized by the Cravatt laboratory (Scripps, La Jolla, CA). Naloxone HCl and clonidine HCl were dissolved in physiological saline (0.9% NaCl) and drug concentrations were calculated using the weight of the salt as used previously (Ramesh et al., 2013, 2011). Morphine sulfate was dissolved in physiological saline (0.9% NaCl) and drug concentrations were calculated using the weight of the base. JZL184, PF-3845, SA-57 and THC were dissolved in ethanol and Emulphor-620 (Rhone-Poulenc, Princeton, NJ) and then diluted with physiological saline to form a vehicle mixture of ethanol, Emulphor-620, and saline in a ratio of 1:1:18. JZL184, PF-3845, and SA-57 were administered via intraperitoneal (i.p.) injection. THC, clonidine, and naloxone HCl were given via subcutaneous (s.c.) injection. All drug injections were administered in a volume of 10 µl per g body mass.

2.3. Pellet implantation surgery

In order to induce opioid dependence, mice were implanted with morphine pellets as previously described (Way et al., 1969). In brief, anesthesia was induced with 2.5% isoflurane, the fur was shaved, the skin was disinfected with a sterile betadine swab (Purdue products, Stamford, CT), and a 1 cm horizontal incision was made in the midscapular region using sterile surgical scissors. A 75 mg morphine sulfate pellet or placebo was inserted subcutaneously, and the incision was closed with a sterile staple. All mice received 2 mg/ml acetaminophen (generic children's liquid acetaminophen, cherry flavored) in their drinking water beginning 1 day prior to surgery. The mice were given a minimum 1 h recovery period in heated home cages after surgery and then returned to the vivarium.

2.4. Conditioned place avoidance

The place conditioning apparatus (MED Associates, St. Albans, VT, USA) was composed of three distinct compartments separated by manual sliding doors. A central grey compartment with a plastic floor separated a black compartment with a floor comprised metal bars (0.31

cm) placed in a parallel series and a white compartment with a metal grid mesh floor (0.625×0.625 cm). The dimensions ($w \times l \times h$) of each compartment were as follows: grey compartment (8.25×12.5×13 cm), black and white compartments (16.5×12.5×13cm).

The conditioning procedure was an unbiased and counterbalanced design (i.e., mice were paired with naloxone on either the black or white side). Mice were handled on three consecutive days prior to start of the experiment to acclimate them to the experimenter. On day 1, and all subsequent days, mice were brought into the test room and acclimated for at least 45 min. The mice were placed gently into the central grey chamber with all the doors pre-opened and mice were allowed to ambulate freely between compartments for 20 min. Med-PC software recorded the time spent in each compartment. Mice were then returned to their home cage. On day 2 (saline conditioning; non-withdrawal day), mice were injected s.c. with saline and placed directly in the chamber designated as the saline side where they remained for 30 min. Mice were returned to their home cage and remained in the laboratory until the second conditioning session of the day in which the same procedure was repeated 4 h later. On day 3, mice were implanted with a 75 mg morphine or placebo pellet under anesthesia. Morphine pellets were implanted after the initial saline (non-withdrawal) conditioning sessions to avoid potential confounds of interpretation. Specifically, in a preliminary study we found that mice implanted with morphine pellets and given a saline conditioning session acquired a conditioned place preference (data not shown). Accordingly, behavioral responses of morphine-pelleted mice given saline and naloxone conditioning sessions could be interpreted as either a preference for morphine or an aversion to withdrawal. On day 5, mice were pretreated either 2 h prior to the first conditioning session (JZL184, PF-3845, SA-57) or 30 min prior to both conditioning sessions (THC, clonidine, morphine). Mice were given a single injection of catabolic inhibitors because endocannabinoid levels remain elevated for up to 8 h (Ahn et al., 2009; Long et al., 2009a), which encompasses the course of each of the daily two conditioning sessions. Our laboratory has previously found that 30 min pretreatment time of THC completely blocks naloxone-precipitated somatic withdrawals signs in morphine-dependent mice (Ramesh et al., 2011). Moreover, the enzyme inhibitors employed produce significant elevations in brain endocannabinoid levels at 2 h and ameliorate naloxone-precipitated somatic withdrawals signs in morphine-dependent mice (Ramesh et al., 2013, 2011). At 48 h post-pellet implantation, mice were injected with either naloxone HCl or saline (placebo+saline controls) and placed into the naloxone-designated compartment for 30 min. The naloxone conditioning procedure was then repeated 4 h later (i.e., 52 h post pellet implantation). This procedure was based on previous reports that investigated nicotine withdrawal CPA in which mice implanted with nicotine Alzet osmotic pumps received two conditioning trials per day (Kota et al., 2007; Merritt et al., 2008). Moreover, in pilot studies, we found that a single trial conditioning did not yield reliable acquisition of morphine withdrawal CPA. During naloxone-precipitated withdrawal conditioning, mice were observed for the presence of jumping behavior, which was scored as a binary event (i.e., present or absent) across both conditioning sessions. Jumping served as a second measure of morphine withdrawal and was used for comparison to CPA. On day 6, mice were tested for the expression of CPA during a 20 min test.

Avoidance scores were calculated by subtracting the amount of time spent on the naloxone paired side during the pre-conditioning test from the time spent on the post-conditioning test. Thus, a negative score indicated that the animal spent less time on that side following the conditioning procedure and this value reflected a change from the animal's initial preference for that side. Negative scores are considered to reflect that the conditioning was aversive.

2.5. Somatic withdrawal signs

A naloxone dose-response experiment was conducted in mice 48 h after implantation of a 75 mg morphine pellet. Somatic withdrawal signs were scored, as previously described (Schlosburg et al., 2009). In brief, mice were placed in white acrylic chambers (20×20 cm), with a clear acrylic front panel and a mirrored back panel for a 30 min acclimation period. The chambers were enclosed in sound-attenuating cabinets that contained an indirect filtered LED light source and fans for air circulation and white noise. The mice were briefly removed from the chambers for naloxone administration and immediately returned to the chambers for a 30 min observation period. Behavior was recorded using a series of Fire-i™ digital cameras (Unibrain, San Ramon, CA), and the videos were saved using the ANY-maze™ video tracking software (Stoelting Co., Wood Dale, IL). Chambers were changed between tests and cleaned at the end of testing with an ammonia based cleaner and left to dry for two days, to allow for odors to dissipate. The recorded videos were randomized and scored by a trained observer, who was blinded with respect to treatment condition. The primary behavioral signs of interest were frequency of jumps and front paw tremors (including single and double paw flutters and twitches, which are not commonly displayed by naïve mice). The occurrence of diarrhea during the testing period was noted. All behaviors were recorded as new incidences when separated by at least 1 s or interrupted by any other normal behavior.

2.6. Statistical Analysis

All data are reported as mean ± SEM or as a percentage of mice exhibiting jumping or presenting with diarrhea. Data were analyzed using either Student's t-test or one-way between measures analysis of variance (ANOVA). Dunnett's test was used to compare drug treatments with vehicle and Newman-Keuls post-hoc test was employed for comparisons between various treatments. In addition, planned comparisons were used to compare differences in naloxone CPA between dependent and non-dependent mice. The percentages of mice exhibiting jumping and diarrhea were analyzed using the z-test of two proportions. ED₅₀ values for naloxone were calculated using a least-squares linear regression analysis. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Naloxone-precipitated morphine withdrawal produces conditioned place avoidance behavior at a dose that does not elicit this effect in non-dependent mice

Initial experiments were designed to determine the optimal dose of naloxone that produced CPA in morphine dependent mice, but not in placebo-pelleted mice. The dose-response relationship of naloxone (0.03, 0.056, and 0.1 mg/kg) was examined in morphine- and

placebo-pelleted mice. Naloxone produced a significant CPA in morphine-pelleted mice [$F(3,74) = 4.3$; $p < 0.01$; Figure 1A] as well as in placebo-pelleted animals [$F(3,80) = 4.3$; $p < 0.01$; Figure 1B]. Post hoc analyses revealed that morphine-pelleted mice challenged with 0.056 or 0.1 mg/kg displayed a significant CPA compared with vehicle, while only 0.1 mg/kg produced a CPA in placebo-pelleted mice. Moreover, 0.1 mg/kg naloxone elicited a greater magnitude of CPA in morphine-pelleted animals (-204 ± 38 s) than in placebo-pelleted mice (-87 ± 20 s). Thus, naloxone (0.056 mg/kg) was selected for all subsequent studies to ensure that CPA occurred in morphine-pelleted mice, only.

3.2. Naloxone precipitates somatic withdrawal signs in mice

In morphine-pelleted mice, naloxone elicited significant jumps [Figure 2A], paw flutters [$F(5,26) = 12.0$; $p < 0.0001$; Figure 2B], head shakes [$F(5,26) = 12.9$; $p < 0.0001$; Figure 2C], and diarrhea [Figure 2D]. The ED_{50} values of naloxone for these measures are shown in Table 1. Naloxone (0.3 and 1 mg/kg) produced significant increases in paw flutters, head shakes, and diarrhea whereas naloxone (0.1, 0.3, and 1 mg/kg) produced significant increases in percentage of mice exhibiting jumping behavior.

3.3. Morphine and clonidine reduce acquisition of naloxone-precipitated morphine withdrawal conditioned place avoidance

As a positive control, morphine (30 mg/kg) was administered s.c. 30 min prior to each naloxone conditioning session. Morphine pretreatment significantly attenuated naloxone-precipitated morphine withdrawal CPA [$F(2,23) = 9.6$; $p < 0.001$; Figure 3A] and withdrawal jumping [$p < 0.01$; Figure 3B]. Similarly, clonidine (0.1 and 0.3 mg/kg) significantly reduced withdrawal CPA [$F(4,69) = 4.5$; $p < 0.001$; Figure 3C], but did not significantly affect withdrawal jumping [Figure 3D].

3.4. JZL184, PF-3845, SA-57 and THC do not produce conditioned place preference or avoidance

To determine if THC or the endocannabinoid hydrolytic enzyme inhibitors would produce CPA or conditioned place preference (CPP) when given alone, we tested the maximal doses used in naloxone-precipitated morphine withdrawal CPA experiments in placebo-pelleted mice. None of the treatments produced CPP or CPA in placebo-pelleted mice [Figure 4; $p = 0.18$].

3.5. Effects of THC and endocannabinoid catabolic enzymes inhibitors on withdrawal jumping and acquisition of CPA

To test if a direct agonist of cannabinoid receptors would reduce acquisition of naloxone-precipitated morphine withdrawal CPA, we tested the phytocannabinoid THC. Naloxone produced significant CPA in morphine-pelleted mice [$F(4,23) = 8.8$; $p < 0.001$; Figure 5A], but THC (1, 3 and 10 mg/kg) did not affect acquisition of CPA. However, THC (3 and 10 mg/kg) significantly reduced naloxone-precipitated morphine withdrawal jumping behavior in mice [Figure 5B].

The MAGL inhibitor JZL184 (4 or 40 mg/kg) administered i.p. 2 h prior to the first conditioning session did not prevent naloxone-precipitated CPA in morphine-pelleted mice

[Figure 5C]. An overall significant ANOVA [$F(3,49) = 7.4$; $p < 0.001$;] was due to differences of each experimental group compared with the placebo-pelleted group. In contrast, 40 mg/kg JZL184 significantly reduced the number of mice that exhibited jumping behavior during the conditioning sessions [Figure 5D].

Similarly, PF-3845 (1, 3 or 10 mg/kg) did not reduce naloxone-precipitated withdrawal CPA [Figure 5E]. Again, the significant ANOVA [$F(4,50) = 7.7$; $p < 0.0001$] was the result of differences between the placebo-pelleted mice and each group of mice implanted with morphine pellets. Furthermore, PF-3845 (10 mg/kg) did not significantly affect the number of mice that exhibited jumping behavior during the conditioning sessions [$p = 0.27$; Figure 5F].

In the final experiment, we tested whether dual inhibition of FAAH/MAGL would block morphine withdrawal in the CPA paradigm. Naloxone administration to morphine-dependent mice produced a robust CPA response [$F(4,53) = 8.0$, $p < 0.0001$; Figure 5G], but SA-57 (1.25, 5, or 12.5 mg/kg) was without effect [$p = 0.58$]. However, SA-57 significantly reduced the number of mice exhibiting withdrawal jumping behavior [Figure 5H].

4. DISCUSSION

The experiments in the present study tested whether inhibition of endocannabinoid catabolic enzymes or THC administration would reduce aversive aspects of morphine withdrawal. This hypothesis was tested using a Pavlovian conditioning procedure in which morphine-dependent mice display a CPA to a compartment associated with naloxone-precipitated withdrawal. Neither THC nor the endocannabinoid catabolic inhibitors significantly reduced morphine withdrawal CPA, though each of these compounds, with the exception of the FAAH inhibitor PF-3845, reduced naloxone-precipitated jumping, which was assessed at the time of conditioning. In contrast, the positive control compounds, morphine and clonidine, prevented acquisition of CPA. Importantly, JZL184, PF-3845, and SA-57 at doses sufficient to block FAAH and/or MAGL activity did not elicit either a place preference or a place aversion in placebo-pelleted mice. These findings are consistent with previous work showing that the FAAH inhibitor URB597 is not self-administered in squirrel monkeys (Justinova et al., 2008). Likewise, URB597 did not enhance brain reward function as assessed in the intracranial self-stimulation paradigm, but actually had an inhibitory effect on this reward process (Kwilasz et al., 2014; Vlachou et al., 2006). Collectively, these findings suggest that inhibitors of endocannabinoid degradative enzymes lack abuse potential. However, considering the non-monotonic nature of drug effects in conditioned place preference, a full dose-response evaluation would be needed to ascertain whether lower doses of the drugs would elicit a CPP.

Here, we utilized a procedure for establishing naloxone-precipitated morphine withdrawal CPA in mice implanted subcutaneously with a morphine pellet. Naloxone (0.056 mg/kg) produced CPA in morphine-pelleted mice, but not in placebo-pelleted mice [Figure 1], consistent with the idea that at this dose mice exhibited aversion to morphine withdrawal. We also examined the dose-response relationship of naloxone to elicit typical somatic withdrawal signs in mice. The dose of naloxone (0.056 mg/kg) that produced maximal CPA

was below the dose required to precipitate somatic withdrawal signs. Thus, naloxone tends to be more potent in producing CPA than somatic withdrawal signs, corroborating a previous report (Frenois et al., 2002).

Morphine prevented the development of morphine withdrawal CPA and blocked naloxone-precipitated jumping in morphine-pelleted mice. Morphine pretreatment represents what could be considered opioid replacement therapy in which a direct mu opioid receptor agonist such as methadone or buprenorphine shows clinical efficacy (Lobmaier et al., 2010). Alternatively, it is plausible that morphine elicited a place preference that offset the aversive state elicited by naloxone in morphine pelleted mice. Morphine (30 mg/kg) also blocked naloxone-precipitated withdrawal jumping. Morphine (25.6 mg/kg) was previously reported to inhibit withdrawal jumping approximately 80% in morphine-dependent mice administered a large dose of naloxone (25 mg/kg i.p.) (Iorio et al., 1975). In another study, 40 mg/kg morphine shifted the naloxone ED₅₀ value for precipitating withdrawal jumping from 0.1 mg/kg to 0.5 mg/kg (Suzuki et al., 1989). The effectiveness of morphine demonstrates that the CPA paradigm is sensitive in detecting reductions in withdrawal responses as would be accomplished using a model of substitution therapy.

As a second positive control, we tested whether clonidine, which is used clinically for the treatment of opioid withdrawal (Gold et al., 1978; Gossop, 1988), would attenuate acquisition of CPA in our procedure. Clonidine (0.1 and 0.3 mg/kg) significantly reduced acquisition of CPA to morphine withdrawal and to our knowledge this is the first time clonidine has been shown to reduce CPA in morphine-dependent mice undergoing withdrawal. Clonidine has previously been shown to reduce CPA to morphine withdrawal in rats (Kosten, 1994; Schulteis et al., 1998a). Interestingly, none of the doses of clonidine tested (0.03–0.3 mg/kg) affected morphine withdrawal jumping. Previous studies reported that clonidine reduced morphine withdrawal-induced body shakes, jumping, and paw tremors (Dehpour et al., 2001; Fielding et al., 1978; Valeri et al., 1989), but others have reported that while clonidine reduced the height of naloxone-precipitated withdrawal jumps, it did not affect the number of jumps (Alguacil et al., 1989). It is plausible that a broader dose range of clonidine may have reduced the incidence of jumping. Moreover, quantifying the number of naloxone-precipitated jumps in the morphine-dependent mice, instead of scoring jumps as a nominal measure, as well as scoring other naloxone-precipitated withdrawal signs may have revealed an effect of clonidine. Also, it would be interesting to examine whether combination of endocannabinoid catabolic inhibitors and clonidine would yield enhanced anti-withdrawal effects in opioid dependent animals. Although cannabinoids receptor agonists and alpha2 adrenergic agonists produce hypotensive effects (Isaac, 1980; Pacher et al., 2005), there are no reports of which we are aware in which FAAH or MAGL inhibitors reduce blood pressure. Thus, it would be interesting to test whether endocannabinoid catabolic enzyme inhibitors would allow for a reduction in the dose of clonidine required to attenuate opioid withdrawal and minimize hypotensive side effects.

JZL184 (40 mg/kg) did not affect acquisition of naloxone-precipitated morphine withdrawal CPA, though it reduced the percentage of mice that jumped, as previously reported (Ramesh et al., 2013, 2011). Additionally, PF-3845 did not affect withdrawal jumping or CPA. It is important to note that PF-3845 has been shown to be less effective than JZL184 in reducing

withdrawal jumping (Ramesh et al., 2011). As previously discussed, it may be that the anti-withdrawal effects of PF-3845 are not detectable when jumps are scored nominally. We also assessed the dual FAAH/MAGL inhibitor SA-57 (Niphakis et al., 2012), which at high doses elevates brain AEA and 2-AG levels, on naloxone-precipitated morphine withdrawal CPA. SA-57 (5 and 12.5 mg/kg) reduced the percentage of mice that jumped during conditioning, but did not affect withdrawal CPA. Previously, we reported that these doses reduced somatic signs of spontaneous withdrawal. Moreover, SA-57 (5 mg/kg) previously did not produce locomotor deficits suggesting that reductions in jumping are unlikely due to non-specific effects on behavior (Ramesh et al., 2013). Finally, we tested the phytocannabinoid THC, an agonist at CB₁ (Matsuda et al., 1990) and CB₂ (Munro et al., 1993) receptors, which is well-established to attenuate opioid withdrawal signs (Bhargava 1976; Hine et al., 1975; Ramesh et al., 2011). THC reduced jumping associated with naloxone-precipitated morphine withdrawal, but did not affect CPA.

The interactions between the endogenous cannabinoid and opioid systems are well known (Vigano et al., 2005) and in terms of opioid withdrawal have recently been reviewed (Scavone et al., 2013). Repeated morphine administration in rats leads to reduced levels of 2-AG in multiple brain regions including striatum, cortex, limbic area, and hypothalamus (Vigano et al., 2003). These findings by Vigano et al. are intriguing considering that in the present study the MAGL inhibitor reduced naloxone-precipitated jumping, while sole FAAH inhibition did not. Similarly, our laboratory previously reported that while full MAGL inhibition reduced all measured somatic withdrawal signs, full FAAH inhibition reduced only a subset of those (Ramesh et al., 2011). Interestingly, full inhibition of FAAH combined with partial inhibition of MAGL yielded enhanced effects in reducing morphine withdrawal in mice suggesting that elevating both AEA and 2-AG can reduce all somatic withdrawal signs when administered together (Ramesh et al., 2013). Nonetheless, naloxone challenge to ICR mice implanted with 75 mg morphine pellets or placebo pellets did not produce detectable changes in either 2-AG or AEA in brain regions implicated in opioid withdrawal (i.e., locus coeruleus, periaqueductal grey, and amygdala; Ramesh et al., 2011).

There are several explanations for the efficacy of THC and inhibitors of endocannabinoid hydrolytic enzymes to reduce morphine somatic withdrawal signs. First, since CB₁ and mu opioid receptors are coupled to G_{i/o} proteins and share common downstream signaling events including mutual inhibition of adenylyl cyclase (Childers et al., 1992) via activation of G_{i/o} proteins (Robledo et al., 2008). Opioid withdrawal involves super-activation of adenylyl cyclase which results in cAMP overshoot (Avidor-Reiss et al., 1995), hyperexcitability (Lane-Ladd et al., 1997; Widnell et al., 1994), and increased noradrenergic outflow (Crawley et al., 1979; Van Bockstaele et al., 2008) from the locus coeruleus. The efficacy of CB₁ receptors agonists to inhibit adenylyl cyclase (Matsuda et al., 1990; Vogel et al., 1993) provides a means by which super-activation of adenylyl cyclase could be throttled since CB₁ receptors are colocalized with mu opioid receptors in locus coeruleus (Scavone et al., 2010). Thus, inhibition of adenylyl cyclase resulting in a reduction in the cAMP overshoot that accompanies opioid withdrawal represents a possible mechanism through which cannabinoids attenuate the physical signs of opioid withdrawal. Second, it has been suggested that CB₁ receptors localized on glutamatergic afferents in the locus coeruleus could further inhibit activation of these neurons (Mendiguren and Pineda, 2007; Scavone et

al., 2013). The present data suggest that activation of CB₁ receptors is insufficient to attenuate acquisition of withdrawal CPA. It may be that cannabinoids and other drugs (e.g., clonidine) differentially affect distinct neural systems mediating the expression of somatic signs and acquisition of withdrawal CPA. While the locus coeruleus appears important for the expression of somatic withdrawal signs, the amygdala and nucleus accumbens have been suggested to be more sensitive to naloxone-precipitated withdrawal and mediate the aversive components (Frenois et al., 2002; Hand et al., 1988; Stinus et al., 1990). Our data are consistent with the idea that different mechanisms contribute to the blockade of somatic withdrawal behaviors and the conditioned aversive aspects of opioid withdrawal. Specifically, morphine reduced both somatic withdrawal signs and withdrawal-mediated CPA, while cannabinoid reduced the former clonidine reduced the latter.

In conclusion, cannabinoid receptor agonism either directly with THC or indirectly via inhibition of endocannabinoid catabolic enzymes does not prevent morphine withdrawal CPA. The CPA paradigm represents one among many assays sensitive to opioid withdrawal, and may reflect an aversive state. For example, opioid withdrawal elevates reward thresholds in intracranial self-stimulation, which may indicate an anhedonia-like effect (Altarifi and Negus 2011; Kenny et al., 2006; Liu and Schulteis, 2004; Schaefer and Michael, 1983, 1986). Furthermore, opioid withdrawal produces anxiogenic effects in rats in the elevated plus maze (Schulteis et al., 1998b; Zhang and Schulteis, 2008). Future studies should examine the effects of cannabinoids on these behaviors related to opioid withdrawal as CPA represents only one measure of aversive aspects of withdrawal, and endocannabinoid catabolic inhibitors may exhibit efficacy in these other measures. Despite the lack of effects on withdrawal CPA, THC, MAGL inhibition, and dual inhibition of MAGL and FAAH reduced somatic withdrawal signs, which represent a significant component of opioid withdrawal. Moreover, the observation that none of the inhibitors tested in non-dependent mice produced a place preference is consistent with the idea that FAAH, MAGL, or combined FAAH and MAGL inhibitors might be used therapeutically, with reduced risk of abuse.

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Highlights

1. Morphine or clonidine reduces the development of conditioned place aversions of withdrawal in morphine-dependent mice.
2. THC and endocannabinoid degradative enzyme inhibitors reduce somatic signs of morphine withdrawal, but not the development of conditioned place aversions of withdrawal.
3. Endocannabinoid degradative enzymes inhibitors do not elicit rewarding effects on their own, as assessed in the conditioned place preference paradigm.

Figure 1.

Naloxone-precipitated morphine withdrawal produced conditioned place avoidance (CPA) behavior in mice. In (A) morphine-pelleted mice, naloxone (0.056 and 0.1 mg/kg) produced a significant CPA behavior whereas in (B) placebo-pelleted mice, only naloxone (0.1 mg/kg) produced CPA. n=16–26 mice per group. Data for placebo-saline control animals are the same in panels A and B. Data are expressed as mean $\pm$ SEM, or as a percent of animals that exhibited the specified behavior. * $p < 0.05$, ** $p < 0.01$ compared to placebo+saline control.

Figure 2.

Naloxone precipitated somatic withdrawal behaviors in mice implanted with 75 mg morphine pellets. Behaviors scored were (A) presence of jumping behavior, (B) paw flutters, (C) head shakes, and (D) presence of diarrhea. $n=5-6$ per group. Data are expressed as mean $\pm$ SEM, or as a percent of animals that exhibited the specified behavior. Saline-treated mice did not exhibit jumping (A) or diarrhea (D). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to saline control mice.

Figure 3.

Morphine and the α_2 adrenergic agonist clonidine blocked naloxone-precipitated morphine withdrawal CPA. Morphine (30 mg/kg) administered 30 min prior to naloxone conditioning blocked withdrawal CPA (**A**) and withdrawal jumping (**B**). Clonidine (0.1 and 0.3 mg/kg) administered 30 min prior to the first conditioning session reduced acquisition of naloxone-precipitated morphine withdrawal CPA (**C**), but did not affect the percentage of mice that exhibited jumping behavior (**D**). $n=6-10$ per group (**A** & **B**), $n=11-20$ per group (**C** & **D**). Data are expressed as mean $\pm$ SEM, or as a percent of animals that exhibited the specified behavior. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to placebo+saline control for (**A** & **C**) and compared to morphine+naloxone for (**B**), # $p<0.05$, ### $p<0.001$ compared with the morphine+naloxone group.

THC (10.0 mg/kg)	-	+	-	-	-
PF-3845 (10.0 mg/kg)	-	-	+	-	-
SA-57 (12.5 mg/kg)	-	-	-	+	-
JZL184 (40.0 mg/kg)	-	-	-	-	+

Figure 4.

THC, PF-3845, SA-57, and JZL184 do not produce conditioned place preference or avoidance on their own in placebo-pelleted mice. When tested in placebo pelleted mice, neither THC (10 mg/kg), PF-3845 (10 mg/kg), SA-57 (12.5 mg/kg) nor JZL184 (40 mg/kg) produced CPP or CPA on their own. $n=7-14$ per group. Data are expressed as mean $\pm$ SEM.

Figure 5.

THC, JZL184, and SA-57 reduced withdrawal jumping but, did not affect acquisition of a CPA in morphine-dependent mice undergoing withdrawal. THC (1, 3, or 10 mg/kg) did not affect acquisition of naloxone-precipitated morphine withdrawal CPA (**A**), but significantly reduced the percentage of mice exhibiting jumping behavior (**B**). JZL184 did not affect acquisition of CPA to naloxone-precipitated morphine withdrawal (**C**), but reduced percentage of mice that exhibited jumping behavior (**D**). PF-3845 (1, 3, or 10 mg/kg) did not affect acquisition of naloxone-precipitated morphine withdrawal CPA (**E**) and did not

significantly reduce the percentage of mice exhibiting jumping behavior (**F**). SA-57 (1.25, 5.0, and 12.5 mg/kg) administered i.p, 2 h prior to the first conditioning session did not affect acquisition of naloxone-precipitated morphine withdrawal CPA (**G**), but reduced the percentage of mice that exhibited jumping behavior (**H**). n=9–17 per group (**A & B**), n=10–12 per group (**C & D**), n=9–12 per group (**E & F**), n=5–6 per group (**G & H**). Data are expressed as mean $\pm$ SEM, or as a percent of animals that exhibited the specified behavior. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to placebo+saline control for (**A, C, E, G**) and compared to morphine+naloxone for (**B, D, F, H**).

Table 1

ED₅₀ values with 95% CLs of naloxone in precipitating somatic withdrawal signs in mice implanted with 75 mg morphine pellets (n = 5–6 mice/group).

Withdrawal Sign	ED ₅₀ (95% CL) mg/kg
Jumps	0.06 (0.03 – 0.13)
Paw flutters	0.24 (0.17 – 0.33)
Head shakes	0.30 (0.22 – 0.42)
diarrhea	0.1 (0.06 – 0.16)