Cannabinoid Modulation of the Behavioral Effects of Morphine

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ABSTRACT

LAURENCE L. MILLER: Cannabinoid Modulation of the Behavioral Effects of Morphine
(Under the direction of Linda A. Dykstra)

Researchers have demonstrated interactions between exogenously administered cannabinoid and opioid ligands on a variety on behavioral endpoints. The following studies extend these findings by examining the role of the endogenous cannabinoid system in the antinociceptive, and other behavioral effects of morphine.

The experiments in Chapter 2 use disruption of signaling via the type 1 cannabinoid receptor (CB1) in order to examine the role of the endogenous cannabinoid system in the antinociceptive effects of morphine. The effects of morphine did not differ in CB1 KO and WT mice; however, a CB1 antagonist did attenuate the effects of morphine. This suggests that endogenous cannabinoid signaling via CB1 receptors modulates the antinociceptive effects of morphine, and CB1 KO mice may undergo developmental changes that mask this role.

The experiments described in Chapter 3 examine the consequences of the enhancement of endogenous cannabinoid signaling on the antinociceptive effects of morphine. This was accomplished by examining morphine alone and in combination with drugs that inhibit the degradation of the endogenous cannabinoid anandamide (AEA). Two well-established preclinical pain models, the hotplate assay and the acetic acid-induced writhing assay, were used in these studies. The results demonstrate that

inhibition of the enzymatic breakdown of endogenous cannabinoids enhances the antinociceptive effects of morphine in the acetic acid-induced writhing assay, but not the hotplate assay.

Chapter 4 assesses morphine in combination with the same pharmacological manipulations of endogenous cannabinoid signaling that were described in Chapter 3. These experiments extend the previous results by examining these drug combinations in assays of pain-suppressed behavior and schedule-controlled behavior. The results provide evidence that, unlike direct CB1 agonists, drugs that alter endogenous cannabinoid levels specifically alter morphine's antinociceptive effects.

The experimental results described in this dissertation suggest that the endogenous cannabinoid system plays a role in the antinociceptive effects of morphine. In addition, these findings show that this role is dependent on variables such as the nature of the noxious stimulus, and the means used to prevent endogenous cannabinoid degradation. Finally, these studies suggest that this enhancement of morphine's behavioral effects is limited to antinociception.

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PREFACE

This dissertation was prepared in accordance with the guidelines set forth by the University of North Carolina Graduate School. This dissertation consists of a general introduction, three chapters of original data, and a general discussion chapter. Each original data chapter includes a unique abstract, introduction, results, and discussion section. A complete list of literature cited throughout the dissertation is included at the end of the document. References are listed in alphabetical order.

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AEA	anandamide
ANOVA	analysis of variance
C	celsius
cAMP	cyclic adenosine monophosphate
CB1	type 1 cannabinoid
CB2	type 2 cannabinoid
CL	confidence limits
cm	centimeters
CNS	central nervous system
DAG	diacylglycerol lipase
ED50	dose required to produce 50% of the maximum possible effect
FAAH	fatty acid amide hydrolase
FR	fixed ratio
g	gram
h	hour
i.p.	intraperitoneal
kg	kilogram
KO	knockout
MAGL	monoacylglycerol lipase
mg	milligram
mGlu	metabotropic glutamate
m	minute

ml	milliliter
MPE	maximum possible effect
NAPE	*N*-acyl-phosphatidylethanolamine
NMDA	*N*-methyl-D-aspartate
oz	ounce
s	second
SEM	standard error of the mean
WT	wild-type

CHAPTER 1

GENERAL INTRODUCTION

Type 1 Cannabinoid Receptors, Anandamide and Related Constituents of the Endocannabinoid System

The cannabinoid system is composed of two confirmed receptors: the type 1 (CB1) and type 2 (CB2) cannabinoid receptors. CB1 receptors are located throughout the nervous system and though CB2 receptors were initially thought to be located only on immune cells (Howlett et al., 2002; Pertwee, 2005), there is some evidence of wider distribution, including in the nervous system (Van Sickle et al., 2005; Beltramo et al., 2006; Gong et al., 2006). CB1 receptors are g-protein-coupled ($G_{i/o}$) receptors that are primarily located presynaptically. Their activation results in decreased cyclic AMP (cAMP) production (Howlett et al., 2002) as well as direct inhibition of voltage-gated calcium channels (Chemin et al., 2001). An important consequence of these effects is retrograde modulation of synaptic activity through inhibition of neurotransmitter release from the presynaptic cell (Shen et al., 1996; Katona et al., 1999). CB2 receptors are coupled to similar signaling mechanisms, but do not alter the functioning of ion channels. Their role in the nervous system is less clear than that of CB1 receptors (Chin et al., 2008).

Anandamide (AEA) and 2-arachidonylglycerol (2-AG) are the most well characterized endogenous cannabinoids. They are not stored in vesicles, but are produced

as a result of activity-dependent post-synaptic calcium increases from the phospholipid precursors, N-arachidonoyl-phosphatidylethanolamine (NAPE) and 1,2-diacylglycerol (DAG), respectively. These post-synaptic calcium increases may come as a result of depolarization or recruitment from intracellular stores (Di Marzo, 2006). AEA has a higher affinity for CB1 than CB2 receptors and acts as a partial agonist. 2-AG binds to both receptors with lower affinity than AEA but acts as a full agonist (Mechoulam et al., 1995; Di Marzo et al., 2005).

After acting at presynaptic CB1 receptors, AEA is taken into the postsynaptic cell and broken down by fatty acid amide hydrolase (FAAH; Gulyas et al., 2004). Monoacylglycerol lipase (MAGL) metabolizes 2-AG into inactive constituents and this occurs presynaptically (for review, Piomelli, 2003; Gulyas et al., 2004). It has been confirmed that enzymatic breakdown of endocannabinoids occurs intracellularly, but there is controversy over whether endocannabinoid transport for this process occurs via an active or passive mechanism (Di Marzo, 2006). As would be predicted based on their relationship with AEA, FAAH and CB1 have complimentary post- and pre-synaptic distribution, respectively, throughout the nervous system (Egertová et al., 2003; Gulyas et al., 2004).

Cannabinoid modulation of nociception

In the late 19th century Dixon (1899) provided some of the first scientific evidence of the antinociceptive effects of cannabinoids when he demonstrated that dogs show a diminished response to pin pricks after inhaling marijuana smoke. Subsequent studies have demonstrated that drugs acting as cannabinoid agonists produce antinociception in

thermal (Lichtman and Martin, 1997; Welch et al., 1998), mechanical (Clayton et al., 2002) and inflammatory (Calignano et al., 1998) acute pain models, as well as models of persistent pain (Tsou et al., 1996; Herzberg et al., 1997). In addition, it has been demonstrated that the administration of cannabinoids suppresses activity in pain circuits in response to a variety (thermal, chemical, and mechanical) of noxious stimuli (Hohmann et al., 1995, 1999; Martin et al., 1996). Research has also revealed that other behavioral effects occur as a result of the administration of cannabinoid agonists. Cannabinoid agonists produce catalepsy, inhibition of locomotor activity, and disruption of thermoregulation (Martin et al., 1991). Assessments of these three characteristics, along with antinociception, have come to be part of a behavioral screen that is known as the "cannabinoid tetrad." Research suggests that these effects are primarily mediated by CB1 receptors.

More recently, manipulations of endogenous cannabinoid activity have garnered interest, and manipulations of AEA activity at CB1 receptors have been studied most extensively. There is evidence that AEA is synthesized in response to exposure to noxious stimuli, and acts at CB1 receptors to modulate pain (Walker et al., 1999; Mitrirattanakul et al., 2006; Jhaveri et al., 2006; Petrosino et al., 2007; Richardson et al., 2008; Kaufmann et al., 2009). When administered systemically, AEA produces antinociception and other tetrad effects (Vivian et al., 1998; Adams et al., 1998); however, relative to exogenous cannabinoids such as Δ^9-THC, these effects are diminished and brief. It is likely that this is due to the rapid uptake of AEA and its subsequent metabolism by FAAH. Consistent with this hypothesis, administration of an AEA reuptake blocker results in accumulation of AEA (Beltramo et al., 1997; Moore et

al., 2005) as well as enhancement of analgesia produced by systemic administration of AEA (Beltramo et al., 1997; Moore et al., 2005; La Rana et al., 2006). In addition, pharmacological inhibition of FAAH elevates AEA levels and produces CB1 dependent antinociception (Kathuria et al., 2003; Lichtman et al., 2004, 2008). Consistent with studies examining pharmacological inhibition of FAAH, FAAH knockout mice, have approximately 10-fold higher AEA levels relative to their WT littermates, have increased sensitivity to the antinociceptive effects of AEA and display reduced baseline pain sensitivity in the tail-immersion, hot plate, formalin and acetic acid models (Cravatt et al., 2001; Lichtman et al., 2004, 2008; Wise et al., 2007).

Interestingly, manipulations such as pharmacological inhibition of FAAH typically produce antinociception in the absence of catalepsy, disruptions of locomotor activity and other effects associated with administration of CB1 agonists (Kathuria et al., 2003; Jayamanne et al., 2006), though there is one report of decreased locomotor activity after the administration the AEA uptake inhibitor, AM404 (Giuffrida et al., 2000).

Evidence of interactions of the cannabinoid and opioid systems

The cannabinoid and opioid systems are comparable in a variety of ways. Both include G-protein-coupled receptors that are similarly distributed throughout the CNS (Mailleux and Vanderhaeghen, 1992; Maekawa et al., 1994; Mansour et al., 1995; Arvidsson et al., 1995; Lichtman et al., 1996; Tsou et al., 1998; Chen et al., 2002; Cristino et al., 2006), and there is evidence of their co-localization (Rodriguez et al., 2001; Salio et al., 2001). Activation of these receptors typically results in decreased synaptic activity (Howlett et al., 2002; Freund et al., 2003; Bodnar, 2009). In addition,

agonists at CB1 and opioid receptors produce similar behavioral effects that include antinociception (Cox and Welch, 2004; Fischer et al., 2008a), disruption of normal locomotor activity (Pascual et al., 2005; Smith et al., 2009), effects on food consumption (Järbe and DiPatrizio, 2005; Li et al., 2006), and interference with thermoregulation (Wang et al., 2008; Diaz et al., 2009). Moreover, agonists of both types of receptors serve as reinforcers in self-administration paradigms (Negus and Rice, 2008; Justinova et al., 2008) and produce conditioned place preference (Braida et al., 2001; Carrigan and Dykstra, 2007).

Researchers have investigated the possibility that these systems interact and a number of studies have examined the analgesic effects of cannabinoid/opioid combinations. It is now well-established that administration of CB1 agonists enhance the antinociceptive effects of opioids (Welch and Stevens, 1992; Smith et al., 1994b, 2007; Pugh et al., 1996; Smith, 1998; Welch and Eads, 1999; Cichewicz and Welch, 2003; Cichewicz and McCarthy, 2003; Roberts et al., 2006; Cox et al., 2007). The potentiation of the antinociceptive effects of opioids is interesting at a basic science level and could also have clinical utility; however, evidence from other studies suggests that interactions between cannabinoid and opioid agonists are not limited to antinociception. Acutely administered CB1 agonists and morphine interact to alter locomotor activity (Ayhan et al., 1979), and repeated administration of a cannabinoid agonist produces behavioral sensitization and cross-sensitization to morphine (Cadoni et al., 2001, 2008). Co-administration of a cannabinoid agonist with morphine enhanced sensitization to morphine-induced locomotor activity, and pre-exposure to a cannabinoid agonist increases morphine self-administration (Norwood et al., 2003). Morphine conditioned

place preference is also enhanced by pretreatment with a CB1 agonist (Manzanedo et al., 2004), as is the reinforcing efficacy of other opioids such as heroin (Solinas et al., 2005). These data suggest that CB1 agonists and opioids interact in a similar manner across a variety of behavioral endpoints.

Findings such as those discussed above have prompted researchers to examine the role of endogenous cannabinoids in the effects of opioids, and recent findings suggest that manipulations of endogenous cannabinoid activity at CB1 receptors have functional consequences on the behavioral effects of opioids. For instance, a selective CB1 antagonist but not a CB2 antagonist blocks morphine-induced antinociception as measured by an inflammatory pain model (Pacheco et al., 2008) and the thermal tail-flick test (Pacheco et al., 2009) in mice. In addition, there is emerging evidence that inhibition of the degradation of AEA might enhance the antinociceptive effects of morphine (Pacheco et al., 2008, 2009; Haller et al., 2008). Interestingly, whereas direct CB1 agonism results in hypothermia, catalepsy, decreases in locomotor activity (Adams et al., 1998) and enhances the reinforcing effects of opioids (Solinas et al., 2005), the disruption of AEA breakdown by FAAH does not (Cravatt et al., 2001; Lichtman et al., 2004, 2004; Solinas et al., 2005; Moore et al., 2005). Thus, the consequences of pharmacological enhancement of AEA activity on morphine's behavioral effects might be determined by the behavioral endpoint that is used to examine this interaction.

As discussed above, studies using pharmacological manipulations of endogenous cannabinoid activity have provided evidence that suggests that endogenous cannabinoids modulate morphine's antinociceptive effects; however the results of studies using genetic manipulations of cannabinoid activity are in conflict with these findings. Specifically,

CB1 knockout mice do not differ from their wild-type littermates with regard to morphine's antinociceptive effects (Ledent et al., 1999; Valverde et al., 2000). On their own, these findings suggest that activity at CB1 is not integral to the antinociceptive effects of morphine. Interestingly, studies using knockout mice have implicated CB1 receptors in morphine's reinforcing (Cossu et al., 2001) and conditioned effects (Martin et al., 2000) again suggesting that the behavioral endpoint used to study these interactions, but also the manner of manipulating cannabinoid signaling, could be a determinant of the role of CB1 activity in the effects of opioids.

Based on such findings, it is important to further clarify the role of endogenous cannabinoid activity in the behavioral effects of morphine. Therefore, the proposed experiments examine the consequences of inhibition and potentiation of endogenous cannabinoid signaling on the effects of morphine. This will be accomplished by using genetic and pharmacological manipulations in combination with behavioral assays that are designed to assess the degree to which these manipulations produce antinociception and affect other behaviors.

Preclinical Assessment of Antinociception

There are ongoing discussions of the nature of pain research and how researchers can evaluate pain and potential therapeutics in more valid ways (Mogil and Crager, 2004, 2005; Vierck et al., 2005; Negus et al., 2006). Among the major concerns is how to minimize inconsistencies between promising results in basic research and the failure of those results to translate to the clinical setting. A strategy aimed at accomplishing this is the development of new preclinical models. Many traditional preclinical pain models rely

on noxious stimulus-induced behaviors as the dependent variable. An example of this is the hotplate assay where the subject, typically a mouse or rat, is placed onto a hot surface and the latency to make a response (e.g. licking of the hind paw or jumping) is used as the dependent variable. If an intervention (e.g. a candidate antinociceptive drug) produces an increase in the latency to perform the response, this may be interpreted as an antinociceptive effect; however, a potential problem with such an interpretation is that, in such models, an intervention that produces sedation or motor impairment may result in effects that are mistaken as antinociception. Researchers are aware of such issues and have attempted to address them by assessing the effects of candidate interventions on other behaviors such as locomotor activity, rotorod performance, and behavior maintained under schedules of reinforcement. Recently, researchers have proposed that models that measure pain-suppressed behaviors might complement assays of pain-elicited behavior. For instance, Stevenson et al. (2006) found that morphine and the antipsychotic, haloperidol, which is known to alter locomotor activity in rodents, decreases acetic acid-induced writhing (a pain-elicited behavior). However, only morphine inhibited acetic acid-suppressed feeding (a pain-suppressed behavior). Therefore, morphine, a known analgesic produced antinociception as defined by both assays, whereas the suppression of writhing produced by haloperidol may have been the result of nonspecific effects such as motor impairment or other effects that influenced the animals' ability to produce the measured response.

This is a consideration that is very relevant to the current topic. As discussed above, opioid and cannabinoid agonists produce effects such as sedation and disruption of motor activity (Gühring et al., 2001; Pascual et al., 2005; Smith et al., 2009) in

addition to their antinociceptive effects, and these effects could be a source of confounds in studies utilizing pain-elicited behaviors alone. Assays measuring pain-suppressed behavior are relatively novel and their utility has not been fully determined, but they may be very useful in attempting to separate the antinociceptive effects of cannabinoids, opioids, and their combination, from nonspecific effects such as motor impairment.

Researchers studying the antinociceptive effects of drugs often use models that examine other behavioral endpoints in concert with preclinical pain assays. One reason for this, as discussed above, is to try to account for possible confounds; however, there is another important rationale for this approach. Numerous studies, including some from our own laboratory, have indicated that behavioral endpoint is an important determinant of the effects of drugs and drug combinations (Stevenson et al., 2003, 2005; Fischer and Dykstra, 2006; Fischer et al., 2008b). Therefore, it is important to study the interactions of the cannabinoid and opioid systems across a variety of behavioral endpoints.

Goals of the Dissertation

The primary goal of this project is to examine the role of the endogenous cannabinoid system in mediating the antinociceptive and other behavioral effects of morphine. A secondary goal is to assess the utility of preclinical pain models that measure pain-suppressed behaviors as the dependent variable. The experiments described here aim to achieve these goals by addressing three specific aims. Specific Aim 1 will utilize CB1 receptor knockout mice to determine the consequences of constitutive CB1 inactivity on the effects of morphine in two models of pain-elicited behavior (the hotplate assay and the acetic acid-induced writhing assay) and two models of pain-suppressed

behavior (acetic acid-suppressed feeding and wheel running). Aim 2 will use assays of pain-elicited behavior to determine if pharmacological inhibition of FAAH or inhibition of AEA reuptake produces antinociception and enhances the antinociceptive effects of morphine in a manner that is comparable to direct CB1 agonism. Aim 3 will determine the consequences of pharmacological inhibition of FAAH and AEA reuptake, alone and in combination with morphine, in assays of pain-suppressed behavior and an assay of schedule controlled behavior. Together, these experiments will extend our knowledge of the role of the endogenous cannabinoid system in the antinociceptive effects of morphine.

Chapter 2

EXPERIMENT 1: Effects of Morphine on Pain-elicited and Pain-suppressed Behavior in CB1 Knockout and Wildtype Mice

ABSTRACT

Rationale Pharmacological manipulations of the type 1 cannabinoid receptor (CB1) suggest a role for CB1 in morphine-induced antinociception, but studies utilizing CB1 knockout (KO) mice do not support this conclusion. Since studies using CB1 KO mice to study morphine's antinociceptive effects have only examined thermal pain models, the current study examines these interactions in models that employ a chemical stimulus.

Objectives To determine whether the findings obtained with thermal pain models extend to models that use a chemical stimulus, the effects of morphine on acetic acid-induced writhing were examined in CB1 KO and wildtype (WT) mice. In addition, behaviors that decrease in response to acetic acid injection (pain-suppressed behaviors), feeding and wheel-running, were examined. Investigations were also carried out in the hotplate assay. Finally, the CB1 antagonist SR141716A was used to determine if there are dissimilar consequences of constitutive and acute inactivity of CB1 on morphine antinociception in these assays.

Results Morphine attenuated acetic acid-induced writhing, and responding in the hotplate assay. Morphine also attenuated the suppression of wheel-running following an injection of acetic acid, but did not block the suppression of feeding. Although morphine's effects

were not altered in CB1 KO mice, SR141716A attenuated the effects of morphine in C57BL/6 mice.

Conclusions The antinociceptive effects of morphine do not differ in CB1 KO and WT mice in preclinical pain models using both thermal and chemical stimuli. Since SR141716A did attenuate the effects of morphine it is possible that CB1 KO undergo developmental changes that mask the role of CB1 in morphine's antinociceptive effects.

INTRODUCTION

The cannabinoid and opioid systems include G-protein-coupled receptors, type 1 cannabinoid (CB1) and μ-opioid receptors, respectively, that have parallel distribution throughout the CNS. The activation of these receptors can produce similar effects such as decreased cyclic AMP and inhibition of synaptic activity (Waldhoer et al., 2004; Pertwee, 2006). Further, agonists of CB1 receptors and μ-opioid receptors produce similar behavioral effects that include antinociception (Cox and Welch, 2004), alterations of locomotor activity (Pascual et al., 2005; Smith et al., 2009), food consumption (Järbe and DiPatrizio, 2005; Li et al., 2006), and thermoregulation (Wang et al., 2008; Diaz et al., 2009). Moreover, agonists at these receptors serve as reinforcers in self-administration paradigms (Negus and Rice, 2008; Justinova et al., 2008).

Numerous studies have examined the interactions of these systems, and antinociception is one area in which interactions have been consistently reported. The cannabinoid agonist delta-9-tetrahydrocannabinol (Δ9-THC) potentiates the effects of morphine in the mouse tail-flick test (Cichewicz and McCarthy, 2003) and the rat paw pressure test (Cox et al., 2007). There is also evidence that endogenous cannabinoids modulate the effects of opioids. The CB1 antagonist AM251 blocks morphine-induced antinociception as measured by an inflammatory pain model (Pacheco et al., 2008) and the tail-flick test in mice (Pacheco et al., 2009). Methylarachidonoylflurophosphate (MAFP), which inhibits the degradation of the endogenous cannabinoids anandamide (AEA), and 2-arachidonoylethanolamine (2-AG), enhances the effects of morphine

(Pacheco et al., 2009), as does administration of AEA in combination with the fatty acid amide hydrolase (FAAH) inhibitor URB597 (Haller et al., 2008).

Although studies utilizing pharmacological manipulations of cannabinoid signaling suggest a role for endogenous cannabinoids in the antinociceptive effects of opioids, the results of research with genetic models do not. Specifically, morphine is equally effective in CB1 knockout (KO) and wildtype (WT) mice in the hotplate and tail immersion assays (Ledent et al., 1999; Valverde et al., 2000). It is noteworthy that a variety of pain models have been used in studies utilizing pharmacological manipulations to examine cannabinoid-opioid interactions (Cox et al., 2007; Pacheco et al., 2008, 2009) and in studies phenotyping CB1 KO mice (Ledent et al., 1999; Zimmer et al., 1999). In contrast, studies assessing the antinociceptive effects of morphine in CB1 knockout mice have only examined behaviors that occur in response to acute presentation of thermal noxious stimuli (Ledent et al., 1999; Valverde et al., 2000).

The mechanisms underlying nociception and antinociception depend on factors such as the modality and duration of noxious stimuli (Mogil, 2009) as well as the behavioral endpoints that are used to measure the pain response (Le Bars et al., 2001). This being the case, the current studies extend the existing research on interactions between the cannabinoid and opioid systems by examining CB1 KO and WT mice in preclinical tonic pain assays that employ chemical noxious stimuli and measure behavioral responses that either decrease or increase in response to exposure to noxious stimuli. Specifically, we examined the effects of morphine on the behavioral consequences of intraperitoneal injection of acetic acid in CB1 KO and WT mice. We

sought to determine the role of the CB1 receptor in the effects of morphine on the writhing response to acetic acid injection, and on behaviors that decrease in response to acetic acid injection, namely feeding and wheel-running (pain-suppressed behaviors). CB1 KO and WT were also used to determine the effects of morphine in a thermal (hotplate) assay for comparison.

In addition to noxious stimulus modality and duration, another variable that might account for the dissimilar results obtained from studies using CB1 KO mice and antagonists to examine the antinociceptive effects of morphine is that constitutive inactivity of CB1 in the KO animals might produce changes that mask the role of this receptor in the effects of morphine. Accordingly, SR141716A, a CB1 antagonist, was used to determine the effects of acute disruption of CB1 activity on morphine antinociception.

METHODS

Subjects

Male and female CB1 KO mice and age-matched WT littermates were used for these experiments (total animal numbers: female KO = 49, male KO = 52, female WT = 51, and male WT = 51). The distribution of males and females was balanced across groups except in instances where there were an odd number of mice, in which case males outnumbered females by 1. Mice were generated on a full C57BL/6 background (Zimmer et al. 1999) and heterozygous breeding pairs were obtained from Virginia Commonwealth University. Due to limited access to CB1 KO and WT mice, male C57BL/6 mice were used in studies examining morphine in combination with

SR141716A. All mice were bred and housed in the animal facilities of the Department of Psychology at the University of North Carolina at Chapel Hill. Mice were group housed after weaning and were subsequently individually housed where specified by the experimental protocols. Average mouse weights at the time of testing were as follows: female KO = 19.89 g, male KO = 24.09 g, female WT = 21.42 g and male WT = 26.73 g. C57BL/6 mice weighed 27.83 g on average

Mice had free access to food and water except where specified by the experimental protocols below. Lights were programmed on a 12 h light/dark cycle with lights off at 7:00am. All experiments took place during the dark cycle. Animal protocols were approved by the institutional animal care and use committee, and the methods were in accord with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996)

Experimental Procedures

Assays of pain-elicited behavior

Hotplate

Mice were group housed and had free access to food and water for these experiments. Prior to testing, mice were habituated to the testing room and handling for two days. On the test day, antinociception was assessed using a hotplate analgesia meter (25.3 X 25.3 cm; Columbus Instruments, Columbus, OH). During the hotplate assay the mouse was placed onto the surface of the apparatus and the latency to lick or flutter the

hind paws, or to jump from the hotplate surface was recorded. Responses were measured to the nearest 0.1 s and predetermined cutoff time of 20 s was defined as the maximum trial duration in order to prevent tissue damage. Immediately following termination of a trial, whether due to an animal's response or the fact that the cutoff time elapsed, mice were removed from the apparatus and returned to the home cage.

Baseline latencies were assessed in CB1 KO and WT mice (n = 12 of each genotype) across a range of hotplate temperatures (44-56±0.1°C). Temperatures were tested in ascending order and 15 m elapsed between trials. One week later the same mice were used to assess the effects of morphine using a hotplate temperature of 56±0.1°C. Responses were measured 30 and 15 m prior to drug administration and the latencies from these trials were averaged to yield one baseline value. During dose-effect determination, cumulative doses of morphine were administered 30 m apart in half log increments. Temperatures and doses were examined in a repeated measures fashion. The effect of each dose of morphine is expressed as the percentage of the maximum possible effect (%MPE): [postdrug latency (s) – baseline latency (s)] / [20 – baseline latency (s)].

To determine the consequences of pretreatment with the CB1 antagonist SR141716A on morphine antinociception, C57BL/6 mice were tested at 56^{o} as described above. Morphine (32.0 mg/kg) and SR141716A (3.0 mg/kg) were administered alone and in combination 30 and 60 m prior to testing, respectively. These doses were selected based on prior work in our laboratory with these compounds in this mouse strain.

Acetic acid-induced writhing

Mice were group housed and had free access to food and water until 12 and 5 h prior to testing, respectively. Mice were habituated to the testing room and handling for two days prior to testing. In addition, mice were given a 30 m session with free access to 32% liquid nutrition (Vanilla flavor, CVS brand) on the day prior to testing. Food and water deprivation and access to liquid nutrition occurred due to the fact that data for acetic acid-suppressed feeding were collected in these animals during the same session (see below).

On the test day, separate groups of mice were injected with saline or morphine (0.1 – 10.0 mg/kg) 45 m prior to the start of the test session (n = 5-6 of each genotype per group). Immediately prior to the test session, mice were injected with saline or 0.56% acetic acid. Following the second injection, mice were immediately placed into clean polycarbonate mouse cages (11.5" X 7.5" X 5"; Allentown Inc, Allentown, NJ) containing dishes of 32% liquid nutrition, and their behavior was videotaped for 30 m. Following the session, the tapes were viewed and the number of writhes was recorded. Writhes were operationally defined as an elongation of the body with simultaneous extension of the hind limbs. The effect of each dose of morphine is expressed as percent inhibition of writhing (% Inhibition): [((writhes in saline treated mice - writhes in drug treated mice) / writhes in saline treated mice) X 100].

To determine the consequences of pretreatment with the CB1 antagonist SR141716A on morphine antinociception in the writhing assay, C57BL/6 mice were tested as described above. Morphine (3.2 mg/kg) and SR141716A (3.0 mg/kg) were administered alone and in combination 45 and 60 m prior to testing, respectively. These

doses were selected based on prior work in our laboratory with these compounds in this mouse strain.

Assays of pain-suppressed behavior

Acetic acid-suppressed feeding

Mice were group housed and had free access to food and water until 12 and 5 h prior to testing, respectively. Mice were habituated to the testing room and handling for two days prior to testing. In addition, 24 h prior to testing, mice were given a 30 m session of free access to approximately 14 ml of 32% liquid nutrition (Vanilla flavor, CVS brand; composition as used: protein = 0.36 g/oz, carbohydrate = 1.6 g/oz, and fat = 0.24 g/oz) in order to reduce the novelty of the substance. During this session, individual mice were placed into bedding-free polycarbonate mouse cages (11.5" X 7.5" X 5"; Allentown Inc, Allentown, NJ) that contained a glass dish containing liquid nutrition and were allowed to explore the testing environment and consume the liquid nutrition.

On the test day, separate groups of mice were injected with saline or morphine (0.1 – 10.0 mg/kg) 45 m prior to the start of the test session (n = 5-6 of each genotype per group). Immediately prior to the test session, mice were injected with 0.56% acetic acid or saline. Following the second injection mice were immediately placed into clean polycarbonate mouse cages (11.5" X 7.5" X 5"; Allentown Inc, Allentown, NJ) containing liquid nutrition and the amount of liquid consumed was measured for 30 m. Consumption was quantified by subtracting the weight of the liquid-containing dishes after the session from the weight obtained prior to the session. Because body size might influence consumption independent of other variables, this value was divided by the

animals' weight (grams of liquid consumed per gram of body weight). In the event of spillage, the data were discarded. The effect of each dose of morphine on pain-suppressed consumption is expressed as percent of non-suppressed consumption (% Control): [(acetic acid-suppressed consumption (g/g) / non-suppressed consumption (g/g)) X 100].

To determine the consequences of pretreatment with the CB1 antagonist SR141716A on morphine antinociception in the pain-suppressed feeding assay, C57BL/6 mice were tested as described above. Morphine (3.2 mg/kg) and SR141716A (3.0 mg/kg) were administered alone and in combination 45 and 60 m prior to testing, respectively. The effects of 3.0 mg/kg SR141716A on non-suppressed feeding were also determined. These doses were selected based on prior work in our laboratory with these compounds in this mouse strain.

Acetic acid-suppressed wheel-running

Mice were grouped housed in standard polycarbonate mouse cages (11.5" X 7.5" X 5"; Allentown Inc, Allentown, NJ) until the start of these experiments. At this time mice were individually housed in polycarbonate cages (14" X 10.5" X 5.5"; Tecniplast USA Inc., Exton, PA) containing running wheels (ENV-044, Med Associates, St. Albans, VT). Testing occurred after three weeks of habituation to handling and acquisition of wheel-running behavior. On the day prior to testing (control session) mice were injected with saline followed 45 m later by a second injection of saline. The next day (test session), 24 h after the control session, separate groups of mice were injected with morphine (0.32 – 3.2 mg/kg) or saline and 45 m later were injected with 0.56% acetic acid or saline (n = 6-7 of each genotype per group). Wheel-running was recorded for 30

m following the second injection (acetic acid or saline) during the control and test sessions. The effect of each dose of morphine on pain-suppressed wheel-running is expressed as percent of non-suppressed wheel-running (% Control): [(acetic acid-suppressed running / non-suppressed running) X 100].

To determine the consequences of pretreatment with the CB1 antagonist SR141716A on morphine antinociception in the pain-suppressed wheel-running assay, C57BL/6 mice were tested as described above. Morphine (1.0 mg/kg) and SR141716A (3.0 mg/kg) were administered alone and in combination 45 and 60 m prior to testing, respectively. The effects of morphine and SR141716A on non-suppressed running were also determined. These doses were selected based on prior work in our laboratory with these compounds in this mouse strain.

Drugs

Morphine sulphate and SR141716A were provided by the National Institute on Drug Abuse (Bethesda, MD, USA). Morphine was dissolved in 0.9% saline and SR141716A was dissolved in a vehicle of 100% ethanol, Alkamuls EL-620 (Rhodia, Cranbury, New Jersey) and saline in a ratio of 1:1:18. Acetic acid was purchased from Fischer Scientific and diluted in 0.9% saline. Morphine and SR141716A were injected subcutaneously and acetic acid was injected intraperitoneally at a volume of 0.1 ml/10 g.

Data Analysis

No sex differences were detected in these studies and all data analyses that follow were conducted with groups collapsed across this variable. Data are presented as raw

values (±SEM) or expressed as mean (±SEM) % MPE, % Inhibition, or % Control depending on the assay (see above). The morphine dose required to produce a 50% maximal effect (ED50) was derived using log-linear interpolation when possible, and differences in morphine potency between genotypes were determined and expressed as a potency ratio with 95% confidence limits. Two-factor analyses of variance (ANOVA) were performed to determine the effects of genotype and dose. Student's t-test were used when appropriate to determine genotypic differences and the effects of SR141716A under control conditions. In studies examining the effects of pretreatment with SR141716A on morphine antinociception, one-way ANOVA with Bonferoni comparisons were used to determine treatment effects. All statistical analyses were conducted with an alpha level of significance set at $p < 0.05$.

RESULTS

Fig. 2.1 (top) shows the response latencies for CB1 KO and WT mice across a range of temperatures (44-56±0.1°C) on the hotplate. Two-factor ANOVA revealed temperature dependent decreases in response latencies [$F(4,110) = 296.66$, $p < 0.05$]. However, there were no genotypic differences in response latencies and there was no interaction between genotype and temperature. Baseline responses at 56^{o} did not differ between assessments during the temperature effect curve or morphine dose-effect curve. Morphine (Fig. 2.1 bottom) produced dose-dependent antinociception on the 56^{o} hotplate [$F(3,66) = 174.18$, $p < 0.05$] in CB1 KO [ED50 (95% CL) = 7.35 (5.99-9.01)] and WT mice [ED50 (95% CL) = 8.37 (6.97-10.05)] but there were no differences in the effects of

morphine between genotypes [potency ratio = 1.11 (0.85-1.45)], and no significant interaction of morphine and genotype.

Fig. 2.2 shows the effects of morphine (32.0 mg/kg) and SR141716A (3.0 mg/kg) alone and in combination in the hotplate assay at 56^{o}. One-way ANOVA revealed a significant effect of treatment [$F(3,32) = 111.02$, $p < 0.05$] and bonferoni comparisons show that SR141716A had no effect on hotplate latency. Morphine produced a 100% maximum possible effect in all animals tested ($p < .05$ vs saline) whereas pretreatment with SR141716A significantly attenuated the effects of morphine ($p < .05$ vs morphine alone), though there was still a significant antinociceptive effect ($p < .05$ vs saline).

Fig. 2.3 (top) shows the number of writhes produced by acetic acid in CB1 KO and WT mice in the absence of morphine. KO mice writhed significantly more [28±4.1] than WT mice [15±4.1] in response to acetic acid [$t(10) = 2.2$, $p < 0.05$]. The raw data (number of writhes; Fig. 2.3 bottom inset) indicate that these genotypic differences persisted when morphine was administered prior to acetic acid [$F(1,50) = 16.47$, $p < 0.05$]. However, when basal genotypic differences were taken into account by expressing the data as percent inhibition of writhing (Fig. 2.3 bottom), morphine dose-dependently inhibited writhing [$F(4,50) = 28.99$, $p < 0.05$] in CB1 KO [ED50 (95% CL) = 0.37 (0.27-0.51)] and WT mice [ED50 (95% CL) = 0.34 (0.17-0.66)] and there were no differences in the effects of morphine between genotypes [potency ratio = 1.13 (0.61-2.13)]; there was no significant interaction of genotype and dose.

Fig. 2.4 shows the effects of 3.2 mg/kg morphine and 3.0 mg/kg SR141716A, alone in and in combination on acetic acid induced writhing. One-way ANOVA revealed

a significant effect of treatment [$F(3.20) = 23.90$, $p < 0.05$] such that SR141716 produced a slight but non-significant increase in writhes. Morphine significantly inhibited writhing ($p < .05$ vs saline) and this effect was attenuated by pretreatment with SR141716A ($p < 0.05$ vs saline).

Fig. 2.5 (top) shows the effects of saline and 1.0 mg/kg morphine on liquid nutrition consumption in CB1 KO and WT in the absence of acetic acid. There was a main effect of genotype [$F(1,21) = 9.16$, $p < 0.05$] where WT mice consumed more liquid nutrition than KO mice, but 1.0 mg/kg morphine had no effect on non-suppressed feeding and there was not a significant interaction between genotype and treatment. Intraperitoneal injection of acetic acid almost completely suppressed consumption in both genotypes [WT: 0.008±0.002 g/g; KO: 0.007±0.001 g/g]. Morphine (Fig. 2.5 bottom) was not effective at attenuating acetic acid-induced suppression of consumption. Table 2.1 shows raw consumption data for each genotype (unadjusted by weight).

Pretreatment with 3.0 mg/kg SR141716A (Fig. 2.6 (top)) decreased consumption of ensure in the absence of acetic acid (i.e. consumption not suppressed by pain; $t (5,5) = 2.38$, $p < .05$). Figure 2.6 (bottom) shows that neither 3.2 mg/kg morphine nor 3.0 mg/kg SR141716A, alone or in combination, altered consumption that was suppressed by acetic acid injection.

Fig. 2.7 (top) shows the effects of saline and 3.2 mg/kg morphine on wheel-running behavior in CB1 KO and WT mice in the absence of acetic acid. There was a main effect of genotype [$F(1,20) = 9.36$, $p < 0.05$] where WT mice displayed higher levels of wheel-running than KO mice, however 3.2 mg/kg morphine had no effect on

non-suppressed wheel-running and there was no significant interaction between genotype and treatment. Fig. 2.7 (bottom inset) shows that intraperitoneal injection of acetic acid completely suppressed wheel-running in both genotypes. Morphine significantly attenuated the suppression of wheel-running by injection of acetic acid [$F(4,53) = 6.03$, $p < 0.05$] and the genotypic differences seen with non-suppressed wheel-running persisted with WT mice displaying higher levels of wheel-running than KO mice [$F(1,53) = 5.10$, $p < 0.05$]; there was no significant interaction of morphine and genotype. When basal genotypic differences in wheel-running were taken into account by expressing wheel-running as percentage of control running, the effects of morphine on acetic acid-suppressed wheel running did not differ between genotypes. Morphine ED50 (95% CL) = 1.72 (0.77-3.79) for KO mice and 1.38 (0.84-2.25) for WT mice [potency ratio = 1.18 (0.51-2.94)].

Fig. 2.8 (top) shows that when administered in the absence of acetic acid, morphine and SR141716A, alone and in combination do not alter wheel running behavior in C57BL/6 mice. The bottom portion of Fig. 2.8 shows the effects of morphine, alone and in combination with SR141716A, on pain-suppressed wheel running. One-way ANOVA revealed a significant effect of treatment [$F(2,19) = 10.44$, $p < 0.05$], such that mice treated with morphine produced significantly more wheel revolutions than mice treated with saline ($p < .05$ vs saline). Pretreatment with SR141716A blocked this effect ($p < 0.5$ vs morphine alone).

DISCUSSION

The primary goal of these experiments was to investigate the role of the CB1 receptor in the antinociceptive effects of morphine in CB1 KO mice. Previous studies have shown that CB1 KO mice do not respond differently than WT mice in models of acute thermal pain. In the present study, we extended these findings by determining the effects of morphine in CB1 KO and WT mice in models examining pain resulting from i.p. injection of acetic acid. These models allow for the assessment of both pain-elicited behaviors (writhing) and pain-suppressed behaviors (food consumption and wheel-running). In addition, we compared the results seen in CB1 KO and WT mice with results obtained with the CB1 antagonist, SR141716A in C57BL/6 mice.

The two genotypes did not differ in their responses on the hotplate across temperatures that ranged from innocuous to noxious (44-56±0.1°C). This suggests that CB1 receptors are not integral to this behavioral response to thermal noxious stimuli. Previous research has revealed mixed results with regard to nociceptive responding in the hotplate assay between CB1 KO and WT mice. In a study utilizing mice on the same background as used in the present study (C57BL/6), CB1 KO mice were found to have increased latencies (interpreted as hypoalgesia) relative to WT mice (Zimmer et al., 1999). Consistent with the data obtained in the present study, studies utilizing mice on a CD1 background showed no differences between genotypes (Ledent et al., 1999; Valverde et al., 2000).

Previous studies have obtained mixed results when examining the effects of CB1 antagonists on responses to acute thermal pain with some studies revealing no effect (Cravatt et al., 2001; Hough et al., 2002) and others suggesting a hyperalgesic effect

(Richardson et al., 1998). The present findings are consistent with the former in that, at the dose tested, SR141716A did not alter hotplate latencies.

That there were no differences between CB1 KO and WT mice in response to morphine in the hotplate assay suggests that the absence of functioning CB1 receptors does not alter the effects of morphine in this context. Pretreatment with SR141716A, however, did produce a modest but significant attenuation of the antinociceptive effects of morphine in the hotplate assay. This finding is consistent with other data that suggest that CB1 receptors do modulate the effects of morphine (Pacheco et al., 2008, 2009). Taken together, these findings suggest that morphine's effects do not differ in WT and KO mice because of developmental changes that have taken place in these mice.

In order to determine if the findings of studies using acute thermal pain extend to other stimulus modalities and durations, experiments were conducted with acetic acid serving as a tonic, chemical noxious stimulus. Acetic acid (0.56%) produced more writhes in CB1 KO mice than in CB1 WT mice, suggesting a role for CB1 receptors in the writhing response to acetic acid. Other findings also suggest a role for CB1 receptors in responses to chemical noxious stimuli. For instance, disruption of FAAH activity by pharmacological or genetic means inhibits acetic acid-induced writhing (Naidu et al., 2010), and inhibition of CB1 signaling results in hyperalgesia in the formalin test (Calignano et al., 1998). On the other hand, studies utilizing CB1 KO and WT mice on a CD1 background (Ledent et al., 1999; Valverde et al., 2000) found no differences between genotypes in the writhing test. At the dose tested in the present experiments, the

CB1 antagonist SR141716A did not alter the number of writhes elicited by acetic acid which is consistent with other findings (Booker et al., 2009).

Morphine dose-dependently decreased the number of writhes that result from intraperitoneal injection of acetic acid, and there were no genotypic differences in the effects of morphine in this assay. This suggests that results obtained in other studies utilizing acute thermal pain to study the antinociceptive effects of morphine in CB1 knockout mice extend to this commonly used model of tonic pain. On the other hand, pretreatment with SR141716A attenuated the ability of morphine to inhibit the writhing response, again suggesting that morphine's effects are modulated by CB1, but this role is masked in CB1 knockout mice.

Nociception elicits pain-related behaviors and suppresses other behaviors. Research has demonstrated the utility of preclinical pain assays that measure pain-suppressed behavior in addition to traditional assays that measure pain-elicited behavior (Martin et al., 2005; Stevenson et al., 2006, 2009; Matson et al., 2007; Pereira Do Carmo et al., 2009; Negus et al., 2010). Consequently, the present studies also used CB1 KO and WT mice to determine the role of the endogenous cannabinoid system in morphine's effects on behaviors that are suppressed by exposure to noxious stimuli.

Consumption of palatable food, a behavior that occurs at a high rate under control conditions, is decreased upon exposure to noxious stimuli (Stevenson et al., 2006). In the present studies, control consumption was decreased in mice lacking CB1. This finding, along with the finding that SR141716A decreased non-suppressed food consumption is consistent with a growing literature indicating that the cannabinoid system is an

important mediator of feeding behaviors. Other studies assessing feeding behavior in CB1 KO mice have revealed results similar to those found here (Cota et al., 2003). Moreover, it has been shown that CB1 agonists and antagonists produce hyperphagia (Miller et al., 2004) and hypophagia (Ward et al., 2009), respectively.

In contrast to findings obtained from studies utilizing C57BL/6J mice (Stevenson et al., 2006), in the current study morphine was not effective at attenuating the suppression of feeding by i.p. injections of acetic acid. It seems unlikely that this result is due to suppression of consumption by morphine as morphine had no effect on non-suppressed consumption. The reasons for the discrepancies between the present studies and the Stevenson et al. study are unclear, but there were numerous differences in experimental parameters between the two studies (e.g. amount of exposure to acetic acid and food) that might contribute to the dissimilar findings.

Wheel-running also occurs at a high rate in rodents, and is susceptible to suppression by noxious stimuli. Under baseline conditions, CB1 KO mice exhibited less wheel-running behavior than WT mice which is consistent with other findings (Dubreucq et al., 2010). The CB1 antagonist CB1 antagonist, SR141716A, did not reduce wheel-running when administered to C57BL/6 mice though it has been shown to do so in rats (Keeney et al., 2008). The findings in CB1 KO mice might suggest a role of the endocannabinoid system in the reinforcing effects of wheel-running, but it might also be the case that the differences seen in the present studies are related to other effects of CB1 knockout. For instance, the decreased caloric intake by CB1 KO mice, demonstrated by

others (Cota et al., 2003) and suggested by the present feeding experiments, might lead to decreased wheel-running behavior.

Unlike the results observed in the pain-suppressed feeding assay, morphine dose-dependently attenuated the suppression of wheel-running behavior after i.p. injection of acetic acid. These findings are consistent with previous reports showing that opioids are effective at blocking decreases in a variety of behaviors that result from exposure to noxious stimuli (Martin et al., 2005; Stevenson et al., 2006, 2009; Matson et al., 2007; Pereira Do Carmo et al., 2009; Negus et al., 2010). This suggests that under these conditions, morphine reduces nociception in a manner that not only decreases reflexive responses to i.p. injection of acetic acid (i.e. writhing), but restores rates of pain-suppressed spontaneous behavior. In addition, 3.2 mg/kg morphine, which produced the peak effect on pain-suppressed wheel-running in CB1 KO and WT mice, had no effect on non-suppressed wheel-running. This suggests that the present results are not due to non-specific increases in wheel-running as a result of morphine administration. As stated above, there were no genotypic differences in the effects of morphine on suppressed or non-suppressed wheel-running. On the other hand, as was the case in the other models used here, SR141716A significantly attenuated the ability of morphine to block the suppression of wheel-running by acetic acid injection, again suggesting a role for CB1 receptors in the antinociceptive effects of morphine in this assay.

The findings of the present studies are consistent with research demonstrating that CB1 KO and WT mice do not respond differently to morphine on endpoints related to antinociception (Ledent et al., 1999; Valverde et al., 2000). Moreover, the findings with

SR141716A are consistent with other studies that demonstrate a reduction of the antinociceptive effects of morphine upon administration of CB1 antagonists (Pacheco et al., 2008, 2009). Combined administration of anandamide and a selective inhibitor of its degradation enhanced the effects of morphine (Haller et al., 2008), and Pacheco et al. (2009) also showed that MAFP, which non-selectively inhibits the catabolism of AEA and 2-AG, enhanced the effects of morphine. These authors also demonstrated that a CB2 antagonist did not alter the effects of morphine and it has been demonstrated that the effects of MAFP are mediated via CB1 (Ates et al., 2003).

In the context of the literature, the present results showing attenuation of morphine's effects by the CB1 antagonist, SR141716A, suggest that endogenous cannabinoids are involved in the antinociceptive effects of morphine in models of acute thermal pain and chemical pain. The finding that morphine-induced antinociception does not differ in CB1 KO and WT mice indicates that the constitutive absence of CB1 signaling may result in compensatory changes that mask this role.

Table 2.1 Effects of morphine on liquid nutrition consumption (ml)

Treatment	KO	WT
Control	1.26 (0.18)	2.05 (0.01)
Saline	0.15 (0.02)	0.22 (0.05)
0.1 mg/kg	0.25 (0.03)	0.22 (0.05)
0.32 mg/kg	0.18 (0.02)	0.25 (0.03)
1.0 mg/kg	0.15 (0.02)	0.47 (0.23)
3.2 mg/kg	0.26 (0.03)	0.58 (0.32)
10.0 mg/kg	0.17 (0.02)	0.23 (0.03)

Fig. 2.1 Hotplate assay for CB1 KO and WT mice. Top, temperature-effect curves (44-56 ^{o}C) in the absence of morphine. Abscissa: hotplate temperature (^{o}C). Ordinate: mean response latency in seconds. Bottom, morphine dose-effect curves (1.0 – 32.0 mg/kg) on 56^{o} C hotplate. Abscissa: dose of morphine in milligrams per kilogram. Ordinate: percent maximum possible effect

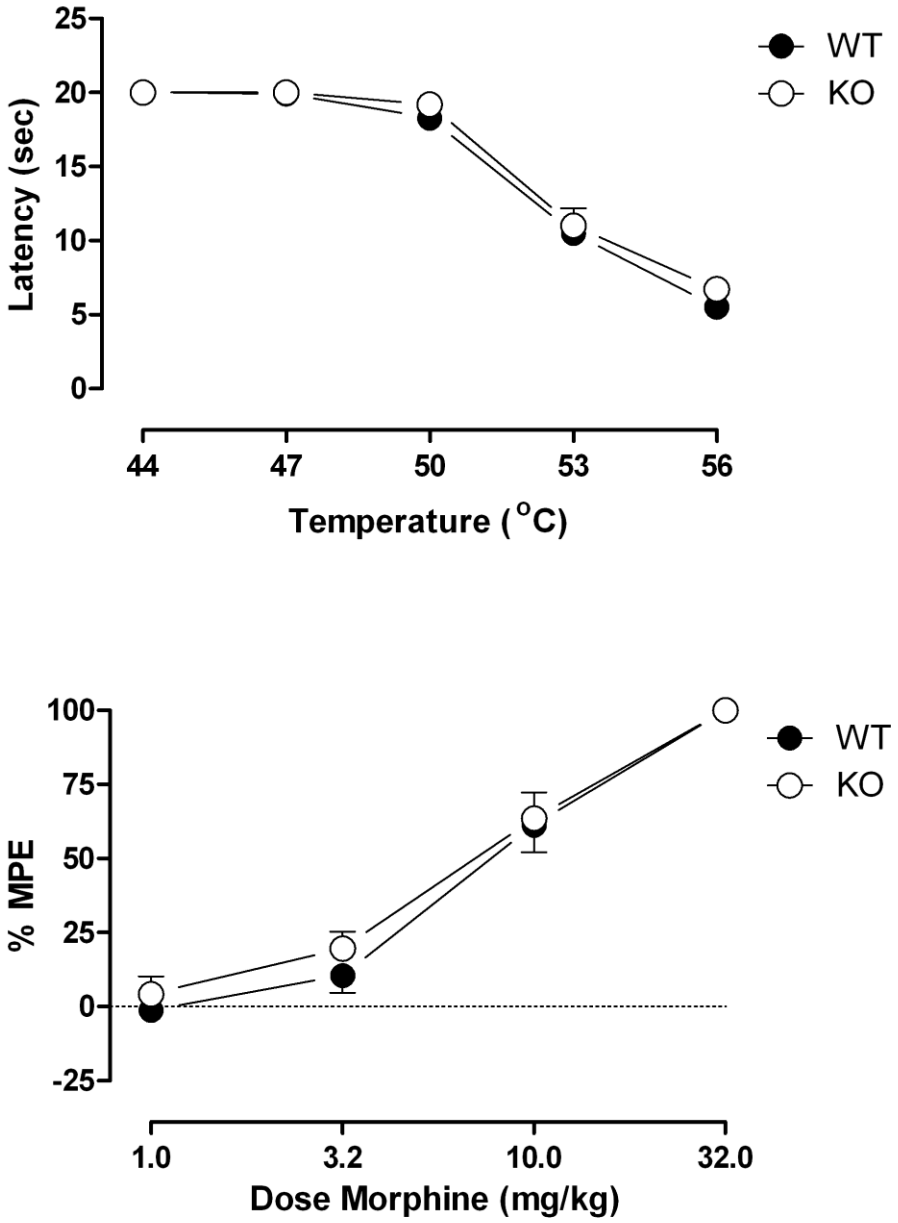


Fig. 2.2 Effect of the CB1 antagonist SR141716A on morphine antinociception in the hotplate assay. Abscissa: treatment. Ordinate: mean response latency in seconds. Asterisk denotes statistical significance compared with saline. Double asterisk denotes statistical significance compared with morphine alone

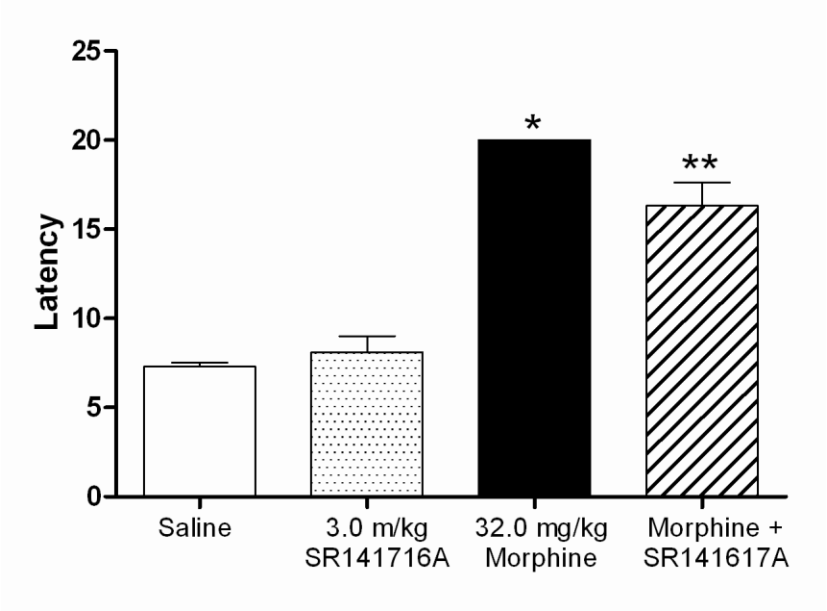
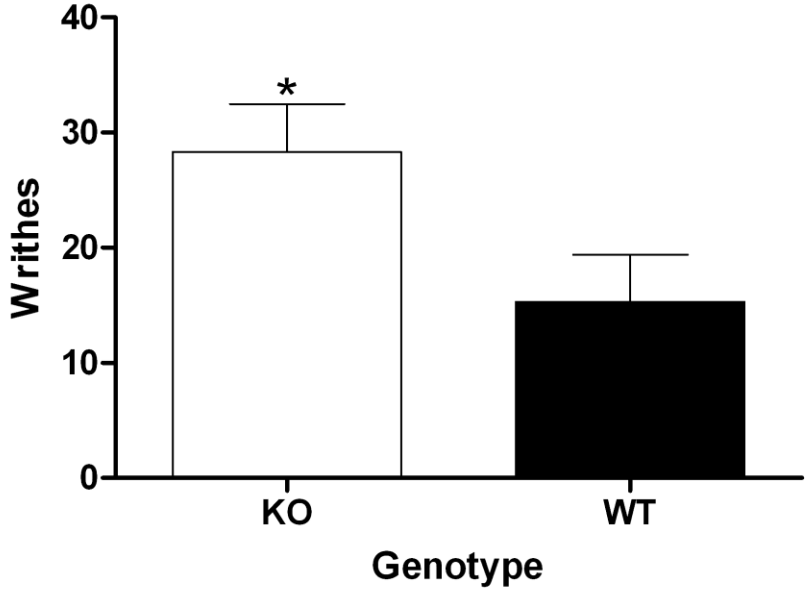


Fig. 2.3 Acetic acid-induced writhing in CB1 KO and WT mice. Top, writhing in response to intraperitoneal injection of 0.56% acetic acid. Ordinate: mean number of writhes. Asterisk denotes statistical significance compared with WT ($p<0.05$). Bottom, effects of morphine on writhing expressed as percent inhibition of writhing. Abscissa: dose of morphine in milligrams per kilogram. Ordinate: percent inhibition of writhing. Bottom inset, effects of morphine on writhing (raw data)



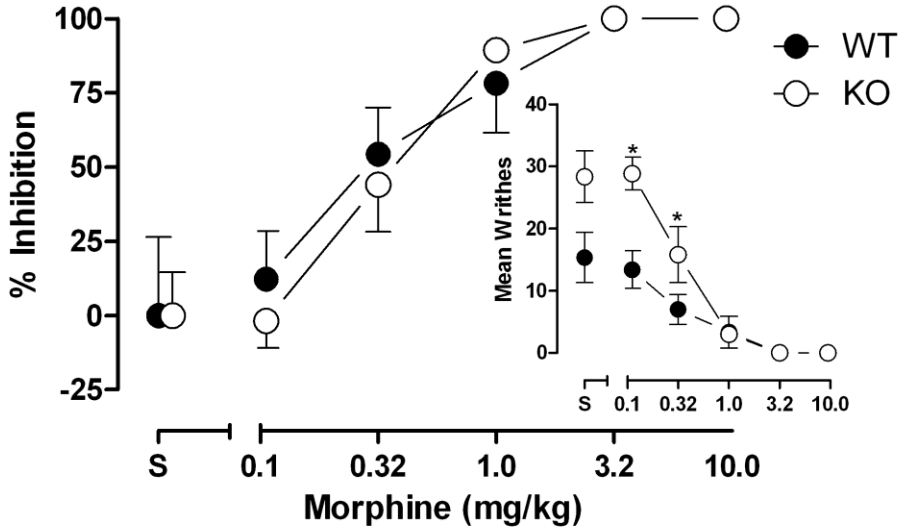


Fig. 2.4 Effect of the CB1 antagonist SR141716A on morphine antinociception in the writhing assay. Abscissa: treatment. Ordinate: mean number of writhes. Asterisk denotes statistical significance compared with saline. Double asterisk denotes statistical significance compared with morphine alone

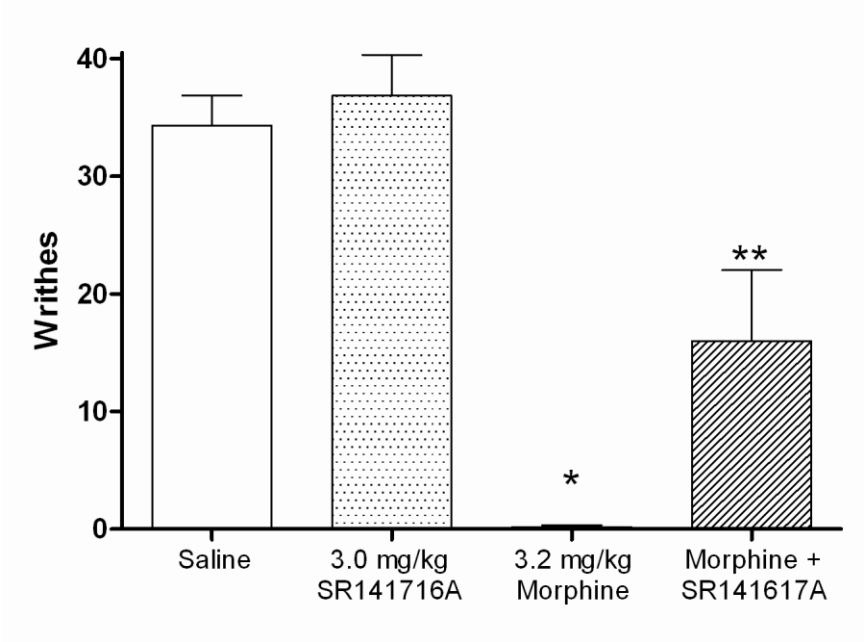


Fig. 2.5 Acetic acid-suppressed feeding in CB1 KO and WT mice. Top, non-suppressed feeding after saline and 1.0 mg/kg morphine. Ordinate: mean grams consumed per gram of body weight. Asterisk denotes statistical significance compared with WT ($p<0.05$). Bottom, effects of morphine on acetic acid-suppressed feeding expressed as percent control. Abscissa: dose of morphine in milligrams per kilogram. Ordinate: percent control consumption. Bottom inset, effects of morphine on acetic acid-suppressed feeding (raw data)

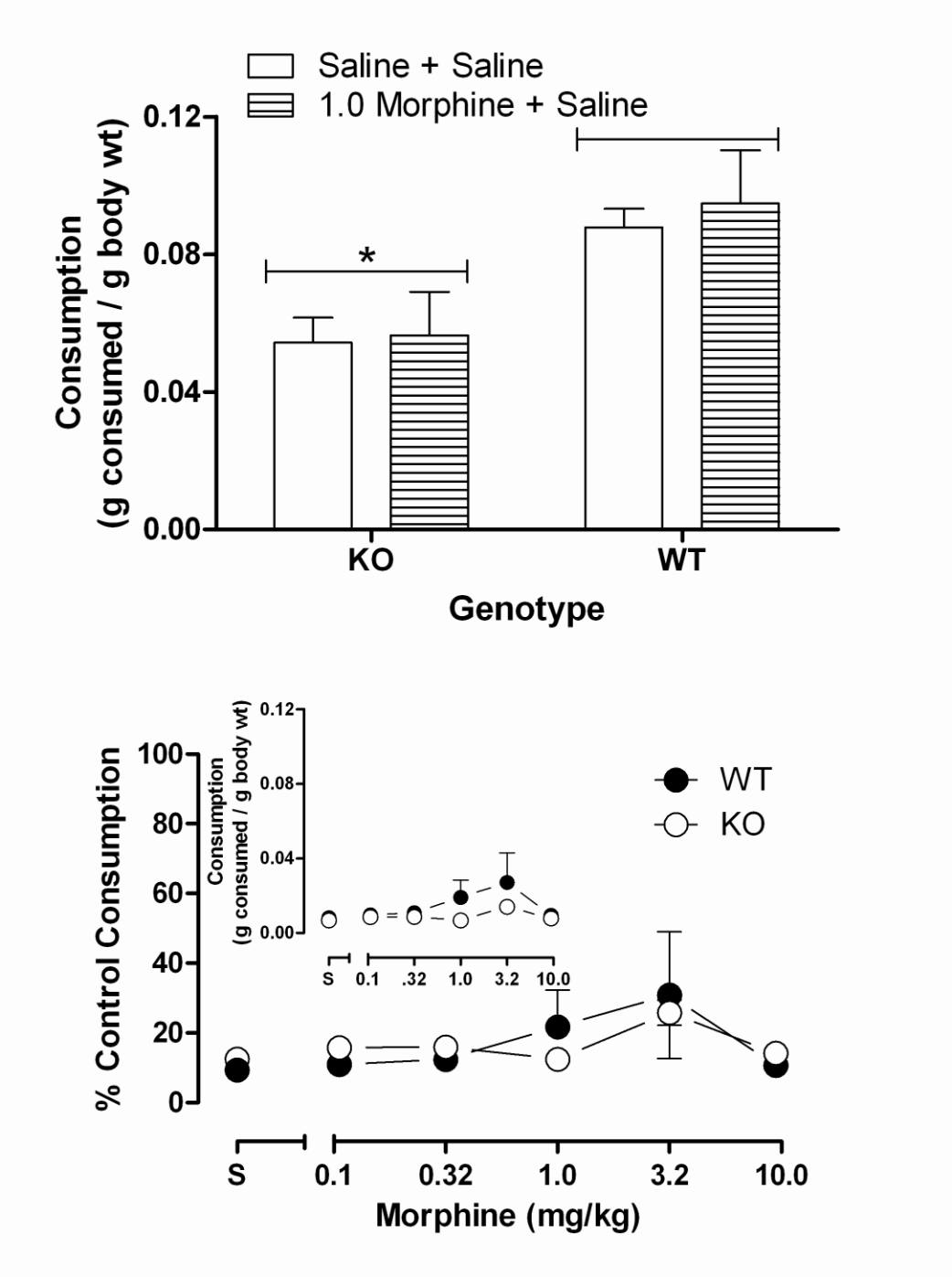


Fig. 2.6 Effect of the CB1 antagonist SR141716A on morphine antinociception in the feeding assay. Top, non-suppressed feeding after saline and 3.0 mg/kg SR141716A. Abscissa: treatment. Ordinate: mean grams consumed per gram of body weight. Asterisk denotes statistical significance compared with saline. Bottom, morphine alone and in combination with SR141716A. Abscissa: treatment. Ordinate: mean grams consumed per gram of body weight

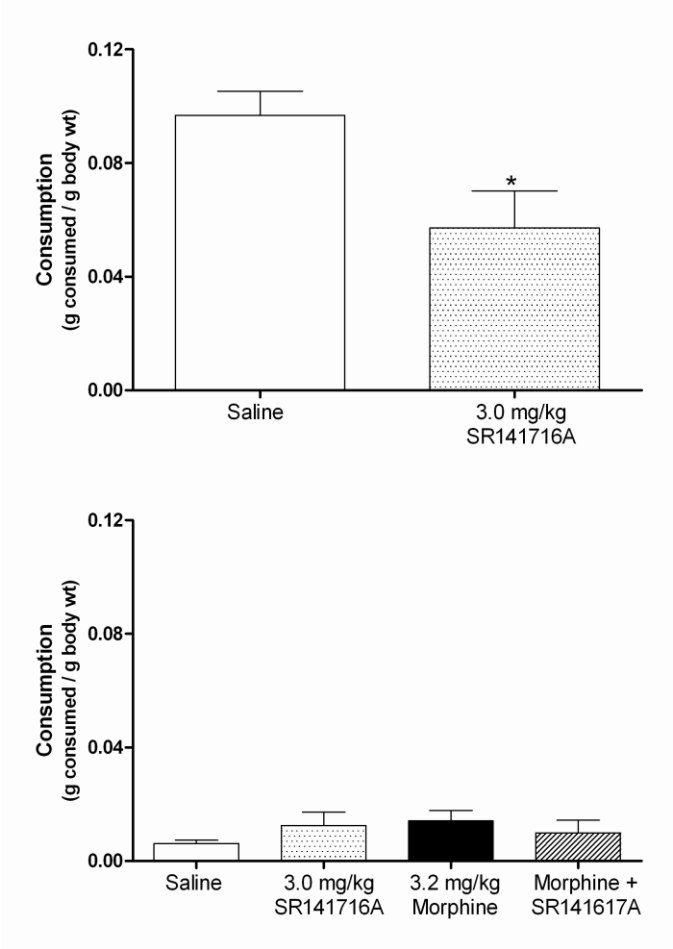


Fig. 2.7 Acetic acid-suppressed wheel-running in CB1 KO and WT mice. Top, non-suppressed wheel-running after saline and 3.2 mg/kg morphine. Ordinate: mean wheel revolutions. Asterisk denotes statistical significance compared with WT ($p<0.05$). Bottom, effects of morphine on acetic acid-suppressed wheel-running expressed as percent control. Abscissa: dose of morphine in milligrams per kilogram. Ordinate: percent control running. Bottom inset, effects of morphine on acetic acid-suppressed wheel-running (raw data)

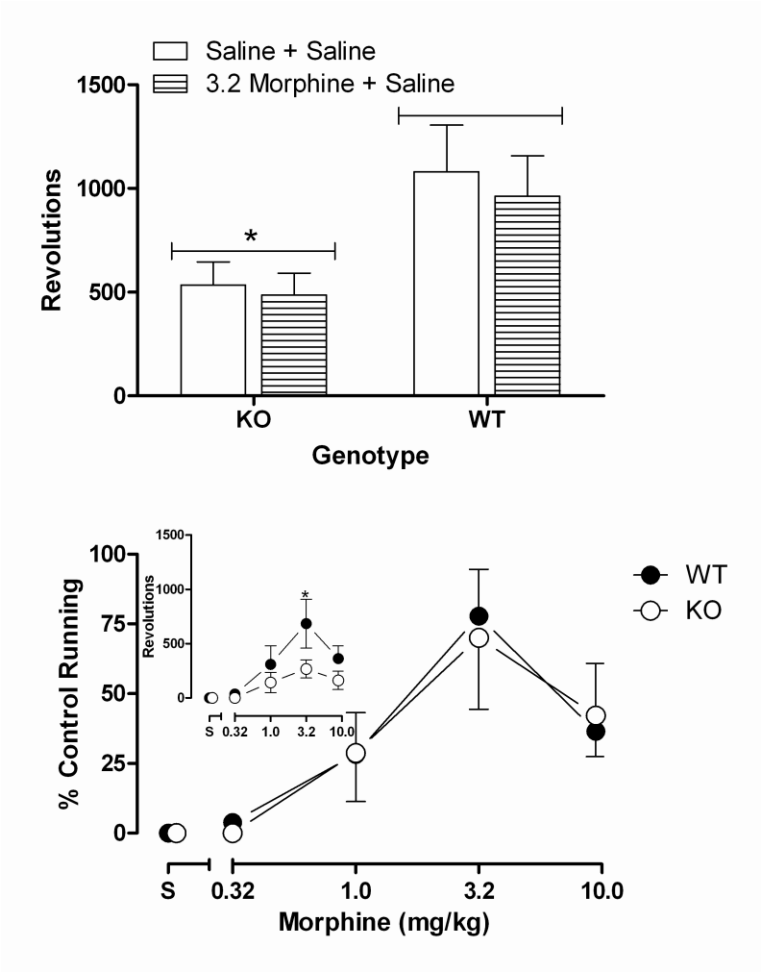
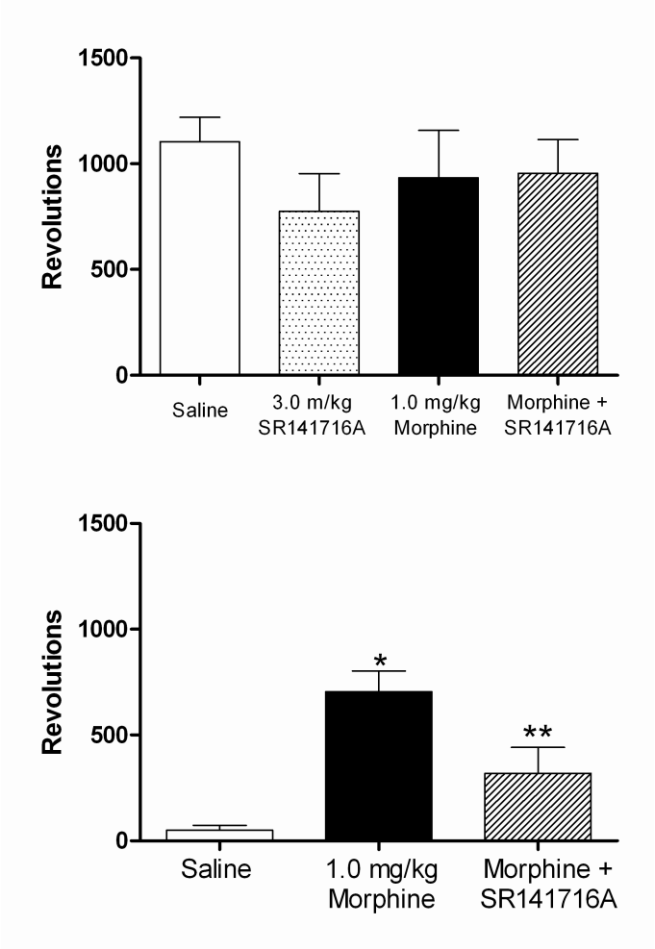


Fig. 2.8 Effect of the CB1 antagonist SR141716A on morphine antinociception in the wheel-running assay. Top, non-suppressed running after saline, 1.0 mg/kg morphine and 3.0 mg/kg SR141716A. Abscissa: treatment. Ordinate: mean wheel revolutions. Bottom, morphine alone and in combination with SR141716A. Abscissa: treatment. Ordinate: mean wheel revolutions. Asterisk denotes statistical significance compared with saline. Double asterisk denotes statistical significance compared with morphine alone



CHAPTER 3

EXPERIMENT 2: Behavioral effects of anandamide modulation and interactions with morphine: pain-elicited behaviors

ABSTRACT

Rationale The endogenous cannabinoid system has received attention from researchers interested in basic pain mechanisms as well as those interested in developing new therapeutics. Drugs that inhibit endocannabinoid degradation produce antinociception in a variety of models, and for the most part, do so without producing other effects associated with CB1 agonists. Recent findings suggesting that endogenous cannabinoids modulate the antinociceptive effects of morphine have further increased interest in this system.

Objectives The FAAH inhibitor, URB597 and the AEA uptake, inhibitor AM404, were examined in two assays of pain-elicited behavior: the hotplate assay and the acetic acid-induced writhing assay. In order to determine if anandamide modifiers produce effects comparable to a direct CB1 agonist, the effects of the CB1 agonist, CP55940 were also examined. In addition, based on evidence that endogenous cannabinoids might modulate the antinociceptive effects of morphine, we tested the hypothesis that blockade of AEA degradation and uptake would enhance morphine's antinociceptive effects.

Results The FAAH inhibitor, URB597 and the AEA uptake inhibitor, AM404, were effective to varying degrees in the writhing assay, but neither had effects in the hotplate

assay. The CB1 agonist CP55940 was effective in both assays, and also enhanced the effects of morphine in both assays. URB597 enhanced the antinociceptive effects of morphine in the acetic acid-induced writhing assay, but did not alter morphine's effects in the hotplate assay. AM404 did not alter morphine's effects in either assay.

Conclusions The present findings are in agreement with the well-established efficacy of CB1 agonists in assays of pain-elicited behavior, as well as findings indicating that CB1 agonists enhance the effects of morphine in these assays. In addition, this study demonstrates that inhibition of FAAH enhances the effects of morphine in a preclinical pain assay that uses a chemical noxious stimulus, which is consistent with recent findings that suggest a role for endogenous cannabinoids in the antinociceptive effects of morphine.

INTRODUCTION

Agonists at type 1 cannabinoid (CB1) and opioid receptors produce similar behavioral effects that include antinociception (Cox and Welch, 2004; Fischer et al., 2008a), disruption of normal locomotor activity (Pascual et al., 2005; Smith et al., 2009), effects on food consumption (Järbe and DiPatrizio, 2005; Li et al., 2006), and interference with thermoregulation (Wang et al., 2008; Diaz et al., 2009). Moreover, agonists of both types of receptors serve as reinforcers in self-administration paradigms (Negus and Rice, 2008; Justinova et al., 2008) and produce conditioned place preference (Braida et al., 2001; Carrigan and Dykstra, 2007).

It is well-established that CB1 and opioid receptor agonists interact in assays of antinociception. For instance, exogenous cannabinoid agonists such as delta-9-tetrahydrocannabinol (Δ9-THC) potentiate the effects of morphine in the mouse tail-flick test (Cichewicz and McCarthy, 2003), the rat paw pressure test (Cox et al., 2007), and in the rat formalin test (Finn et al., 2004). Such interactions are interesting due to their therapeutic potential in the treatment of pain (Welch, 2009), but enthusiasm is tempered by the fact that CB1 agonists also interact with opioids to potentiate effects on other endpoints. This includes the potentiation of opioid effects in assays examining alterations locomotor activity (Finn et al., 2004), as well as in paradigms designed to examine the abuse potential of drugs (Norwood et al., 2003; Manzanedo et al., 2004; Solinas et al., 2005).

Recent research implicates the endogenous cannabinoid system in mediating pain responses. Genetic (Cravatt et al., 2001; Lichtman et al., 2004) and pharmacological

(Lichtman et al., 2004; Costa et al., 2006, 2010) manipulations that inhibit the activity of FAAH or inhibit AEA uptake result in increased levels of AEA that are accompanied by antinociception that is dependent on CB1 receptors. Interestingly, this effect is typically not accompanied by catalepsy, hypothermia and other effects that are associated with the administration of CB1 agonists (Kathuria et al., 2003; Jayamanne et al., 2006).

The results of more recent studies indicate that endogenous cannabinoids might modulate the antinociceptive effects of opioids. For instance, CB1 antagonists attenuate morphine-induced antinociception in the hotplate and writhing tests (Miller et al., under review), the tail-flick test (Pacheco et al., 2009) and hyperalgesia models (Pacheco et al., 2008). In addition, exogenously administered AEA, in combination with the FAAH inhibitor URB597 enhances the antinociceptive effects of morphine in the tail-flick test (Haller et al., 2008). Moreover, methylarachidonoylflurophosphate (MAFP), which inhibits the degradation of the endogenous cannabinoids AEA and 2-arachidonoylethanolamine (2-AG), enhances the antinociceptive effects of morphine in the tail-flick test and in hyperalgesia models (Pacheco et al., 2008, 2009).

Based on these findings, the present study has two goals. First, in order to compare the effects of a FAAH inhibitor, URB597 and an AEA uptake inhibitor, AM404, with a direct CB1 agonist, CP55940, dose-effect curves for each drug were determined in two preclinical pain models: the hotplate assay and the acetic acid-elicited writhing assay. In addition, the role of CB1 receptors in the effects of these drugs was examined by the administration of the CB1 antagonist, SR141716A. Second, based on evidence that endogenous cannabinoids modulate the antinociceptive effects of morphine we tested the

hypothesis that blockade of AEA degradation and uptake would enhance morphine's antinociceptive effects in these models.

METHODS

Subjects

Male C57BL/6 mice purchased from the Jackson Laboratory and bred in house were group housed in the animal facilities of the Department of Psychology at the University of North Carolina at Chapel Hill. Mice of approximately 12 weeks of age at the beginning of testing were used in these experiments, and had free access to food and water except where specified by the experimental protocols below. Lights were programmed on a 12 h light/dark cycle with lights off at 7:00am. All experiments took place during the dark cycle. Animal protocols were approved by the institutional animal care and use committee, and the methods were in accord with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996)

Experimental Procedures

Hotplate

Mice were group housed and had free access to food and water for these experiments. Prior to testing, mice were habituated to the testing room and handling for two days. On the test day antinociception was assessed using a hotplate analgesia meter (25.3 X 25.3 cm; Columbus Instruments, Columbus, OH). During the hotplate test the mouse was placed onto the surface of the apparatus and the latency to lick or flutter the

hind paws, or to jump from the hotplate surface was recorded. Responses were measured to the nearest 0.1 s and predetermined cutoff time of 20 s was defined as the maximum trial duration in order to prevent tissue damage. Immediately following termination of a trial, mice were removed from the apparatus and returned to the home cage.

Drug effects were determined at a hotplate temperature of 56±0.1°C. Responses were measured 30 and 15 m prior to drug administration, and the latencies from these trials were averaged to yield one baseline value. During dose-effect determination for morphine (0.32-32.0) and CP55940 (0.032-3.2), cumulative doses were administered 30 m apart in half log increments. For URB597 (0.1-10.0) and AM404 (0.32-10.0) doses were administered acutely and time-courses were determined. In experiments examining the cannabinoid compounds in combination with morphine, ineffective doses of CP55940, URB597, and AM404 were administered 15 m prior to the commencement of cumulative dosing of morphine as described above. In all experiments, when CP55940, URB597, or AM404 produced a significant effect or produced changes the morphine dose-effect curve, the effects of pretreatment with the CB1 antagonist SR141716A were determined. In this assay, 3.0 mg/kg SR141716A was administered 75 m prior to testing. Our laboratory has previously demonstrated that this dose of SR141716A does not alter hotplate latencies under these conditions (Miller et al., under review). The effect of each treatment is expressed as the percentage of the maximum possible effect (%MPE): [postdrug latency (s) – baseline latency (s)] / [20 – baseline latency (s)].

Acetic acid-induced writhing

Mice were group housed and had free access to food and water until 23 and 5 h prior to testing, respectively. Mice were habituated to the testing room and handling for two days prior to testing. In addition, mice were given a 30 m session of access to 32% liquid nutrition (Vanilla flavor, CVS brand) on the day prior to testing. Food and water deprivation and access to liquid nutrition occurred due to the fact that data for acetic acid-suppressed feeding were collected in these animals, during the same session (see Experiment 3).

On the test day, separate groups of mice were injected with saline or morphine (0.1 – 3.2 mg/kg; 45 m prior to the session), CP 55940 (0.01-0.32; 1 h prior to the session), URB597 (0.32-10.0; 1 h prior to the session), and AM404 (1.0-10.0; 1 h prior to session). In experiments examining the cannabinoid compounds in combination with morphine, the pretreatment times above were maintained and ineffective doses of CP55940, URB597 and AM404 were administered. In experiments examining the effects of SR141716A, 3.0 mg/kg of the antagonist was administered 75 m prior to testing. Immediately prior to the session, mice were injected with 0.56% acetic acid or saline in control experiments. Following this injection, mice were immediately placed into clean polycarbonate mouse cages (11.5" X 7.5" X 5"; Allentown Inc, Allentown, NJ) containing dishes of 32% liquid nutrition, and their behavior was videotaped for 30 m. Following the session, the tapes were viewed and the number of writhes was recorded. Writhes were operationally defined as an elongation of the body with simultaneous extension of the hind limbs. The effect of each treatment is expressed as percent inhibition of writhing (% Inhibition): [((writhes in saline treated mice - writhes in drug treated mice) / writhes in saline treated mice) X 100].

Drugs

Morphine sulphate, URB597 and SR141716A were provided by the National Institute on Drug Abuse (Bethesda, MD, USA). AM404 was purchased from Tocris (Ellisville, MO) and CP 55940 was purchased from Sigma Aldrich (St. Louis, MO). Acetic acid was purchased from Fischer Scientific (Pittsburgh, PA) and diluted in 0.9% saline for intraperitoneal administration (0.1 ml/10g). Morphine, CP55940, URB597, AM404, and SR141715A were injected subcutaneously at a volume of 0.1 ml/10 g.

Data Analysis

Data are expressed as mean (±SEM) % MPE or % Inhibition, depending on the assay (see above). The dose required to produce a 50% maximal effect (ED50) was derived using log-linear interpolation when possible, and differences in potency were determined and expressed as a potency ratio with 95% confidence limits. In instances in which determination of ED50s was not possible, one- and two-way ANOVAs were used to determine drug effects. In these instances, comparisons were conducted using Fisher's protected least significant difference test. All statistical analyses were conducted with an alpha level of significance set at $p < 0.05$.

RESULTS

Fig. 3.1A shows the effects of morphine, CP55940, URB597 and AM404 in the hotplate assay. Morphine [ED50(95%CL) = 9.03(7.04-11.59)] produced dose-dependent increases in %MPE, as did CP55940 [ED50(95%CL) = 0.49(0.30-0.81)]. URB597 and AM404 did not produce antinociception in the hotplate assay (data shown are from the 1

h time point, consistent with the following experiments). As shown in Fig. 3.1B, The CB1 antagonist, SR141716A significantly attenuated the effects of CP55940 [$t(14) = 8.03$, $p < 0.05$].

Fig. 3.2A shows that pretreatment with 0.1 mg/kg CP55940, which was ineffective when administered alone in this assay, produced a significant leftward shift in the morphine dose-effect curve and this effect was attenuated by SR141716A. Neither URB597 nor AM404 altered morphine's antinociceptive effects in the hotplate assay (Fig 3.2B and 3.2C; Table 3.1).

Fig. 3.3A shows that Morphine, CP55940, URB597 and AM404 inhibited acetic acid-induced writhing to varying degrees. These experiments yielded ED50(95%CL) values of 0.42(0.28-0.65) for morphine, 0.03(0.01-0.08) for CP55940, and 1.30(0.92-1.84) for URB597. AM404 partially, but significantly inhibited the writhing response [$F(3,22) = 3.37$, $p < 05$] at doses of 3.2 and 10.0 mg/kg. Fig.3.3B, C, and D shows that 3.0 mg/kg SR141716A significantly attenuated the effects of CP55940 [$t(9) = 6.8$, $p < 0.05$], URB597 [$t(12) = 4.5$, $p < 0.05$], and AM404 [$t(10) = 2.3$, $p < 0.05$].

Fig. 3.4A and 3.4B shows that pretreatment with 0.01 mg/kg CP55940 and 0.32 mg/kg URB597, neither of which was effective when administered alone, produced significant leftward shifts in the morphine dose-effect curve in the writhing assay. ED50(95%CL) and potency ratios for these combinations are shown in table 3.2. In addition, 3.0 mg/kg SR141716A attenuated the effects of 0.32 mg/kg morphine in combination with CP55940 [$t(11) = 2.2$, $p < 0.05$] and URB597 [$t(11) = 2.2$, $p < 0.05$]. AM404 did not alter morphine's effects in this assay (Fig. 3.4C; Table 3.2).

DISCUSSION

The primary findings of this study were that the FAAH inhibitor, URB597 and the AEA uptake inhibitor, AM404, were effective to varying degrees at inhibiting the writhing response to an acetic acid injection, but did not alter response latencies in the hotplate assay. On the other hand, the CB1 agonist CP55940 was effective in both assays. In addition, whereas CP55960 enhanced morphine's antinociceptive effects in both assays, URB597 only enhanced morphine's antinociceptive effects in the writhing assay, and AM404 did not alter morphine's antinociceptive effects at all.

The results observed with CP55940 are consistent with well-established findings demonstrating the efficacy of CB1 agonists in pain assays that use a variety of noxious stimuli (Lichtman and Martin, 1991a, 1991b; Welch et al., 1998). On the other hand, the present findings suggest that antinociception resulting from administration of URB597 and AM404 may be dependent on the assay and/or nature of the noxious stimulus used to examine the drugs' effects. Recent work with a peripherally acting FAAH inhibitor, URB937, may provide insights regarding these differences (Clapper et al., 2010). The Clapper et al. study showed that inhibition of AEA breakdown can produce peripheral antinociception in the writhing model as well as other neuropathic and inflammatory pain models, but does not alter latencies in the hotplate assay. The present studies did not use peripherally restricted drugs, but along with the findings by Clapper et al. (2010) and studies demonstrating that inflammatory stimuli produce site-specific elevations in AEA (Mitrirattanakul et al., 2006; Agarwal et al., 2007), these findings suggest that pharmacological enhancement of AEA activity may be amplified in the presence of

inflammatory and/or tonic noxious stimuli. The amplification of an endogenous response to one type of noxious stimulus (i.e. acetic acid) but not another (i.e. acute thermal pain) by URB597 and AM404 may provide an explanation for the effects observed with these drugs in the present studies.

Previous studies have examined the interaction of the cannabinoid and opioid systems by administering CB1 agonists in combination with morphine and have consistently shown that CB1 agonists enhance the antinociceptive effects of morphine in assays of pain-elicited behavior (Smith and Martin, 1992; Welch and Stevens, 1992; Smith et al., 1994b; Welch et al., 1995; Massi et al., 2001). More recent studies showing that CB1 antagonists attenuate the antinociceptive effects of morphine, provide evidence that endogenous cannabinoids are involved in the antinociceptive effects of morphine (Miller et al., under review; Pacheco et al., 2008, 2009). Moreover, morphine's antinociceptive effects are enhanced when the metabolism of AEA is inhibited (Pacheco et al., 2008, 2009; Haller et al., 2008).

Consistent with these findings, doses of CP55940 that were ineffective when administered alone, enhanced morphine's antinociceptive effects in the hotplate and writhing assays, and a dose of URB597 that was ineffective when administered alone, enhanced morphine's antinociceptive effects in the writhing assay. These enhancements appear to be CB1 receptor-mediated since they were blocked by the CB1 antagonist SR141716A. Interestingly, URB597 did not alter morphine's antinociceptive effects in the hotplate assay, and AM404 did not alter morphine's effects in either assay.

The influence of noxious stimuli on the interaction of URB597 and morphine may be related to the mechanisms discussed above regarding the different effects of URB597 and AM404 in the hotplate and writhing assays when administered alone. In the case of URB597, the enhancement of AEA levels may only be sufficient to alter morphine's antinociceptive effects when AEA levels are already elevated as part of an endogenous response to specific noxious stimuli (i.e. acetic acid). Interestingly, opioid administration has been shown to alter AEA levels as well (Vigano et al., 2004); however, the influence of opioid administration, inflammatory noxious stimuli, and disruption of FAAH degradation, in combination, on AEA levels has not been determined.

The fact that AM404 did not alter morphine's antinociceptive effects is in contrast to our findings with URB597 and other studies that demonstrate that enhancement of AEA levels can alter the antinociceptive effects of morphine (Pacheco et al., 2008, 2009; Haller et al., 2008). Though the exact mechanism is unknown, as an AEA uptake inhibitor, AM404 enhances AEA levels via a mechanism that is distinct from FAAH inhibitors like URB597. Nonetheless, AM404 and URB597 are capable of producing similar changes in AEA levels, in vivo, at the doses and time courses used here (Fegley et al., 2004a, 2005). AM404 also serves as a substrate for FAAH (Fegley et al., 2004a) and binds to, but does not activate CB1 (Khanolkar et al., 1996; Beltramo et al., 1997). Effects beyond those on the accumulation of AEA may contribute to the differing effects of AM404 and URB597 observed here.

These findings show that pharmacological inhibition of FAAH and to a lesser extent, prevention of AEA uptake, result in antinociception in the acetic acid-induced

writhing assay that is mediated at least in part by CB1 receptors. In addition, these experiments show that inhibition of FAAH enhances morphine's antinociceptive effects in this model. These studies, together with other recent findings, suggest that FAAH inhibition may increase AEA levels to a level that produces antinociception and enhancement of opioid antinociception in a manner that is dependent on the nature of noxious stimuli that are employed.

Table 3.1 ED50 (95%CL) values and potency ratios resulting from combined administration of morphine with CP55940, URB597, and AM404 in the hotplate assay.

Hotplate assay. Morphine in combination with CP55940, URB597, and AM404

	ED50(95%CL)	Potency Ratio (95%CL)
Morphine	9.03(7.04-11.59)	-
+ 0.032 CP55940	9.83(7.90-12.23)	1.09(0.80-1.49)
+ 0.1 CP55940	2.60(1.65-4.08)	3.56(2.14-5.92)*
+ 1.0 URB597	6.55(4.94-8.69)	1.31(0.93-1.85)
+ 3.2 URB597	7.67(5.87-10.02)	1.17(0.83-1.64)
+ 10.0 URB597	8.79(6.62-11.67)	1.02(0.72-1.45)
+ 3.2 AM404	6.61(4.77-9.16)	1.28(0.89-1.85)
+ 5.6 AM404	5.92(4.01-8.75)	1.41(0.96-2.08)
+ 10.0 AM404	6.25(4.76-8.22)	1.38(0.99-1.94)
+ 17.0 AM404	8.87(7.19-10.94)	1.03(0.75-1.41)

Table 3.2 ED50 (95%CL) values and potency ratios resulting from combined administration of morphine with CP55940, URB597, and AM404 in the writhing assay.

Writhing assay. Morphine in combination with CP55940, URB597, and AM404

	ED50(95%CL)	Potency Ratio (95%CL)
Morphine	0.42(0.28-0.65)	-
+ 0.01 CP55940	0.16(0.09-0.29)	2.77(1.62-4.82)*
+ 0.32 URB597	0.17(0.10-0.28)	2.44(1.49-4.02)*
+ 1.0 AM404	0.38(0.19-0.76)	1.12(0.64-1.95)

Fig. 3.1 Effects of CP55940, URB597, AM404 and morphine in the hotplate assay. Ordinate: % maximum possible effect. Abscissa: **A** dose (mg/kg) of each drug administered alone. Data points above V/S represent vehicle and saline. **B** Dose (mg/kg) of CP55940 in combination with SR141716A.

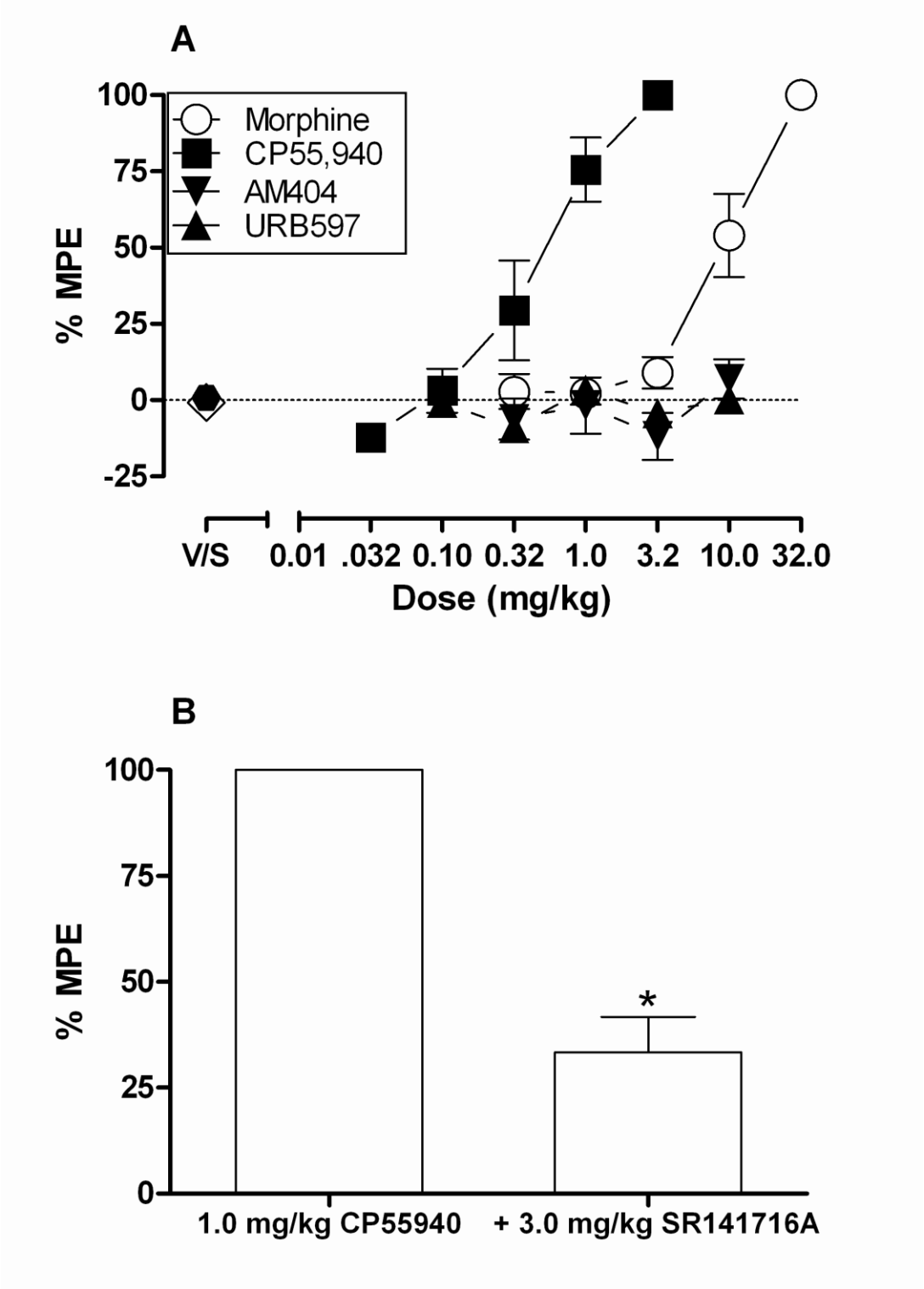


Fig. 3.2 Effects of morphine alone and in combination with CP55940, URB597 and AM404 in the hotplate assay. Ordinate: % maximum possible effect. Abscissa: **A** Dose (mg/kg) of morphine administered in combination with 0.1 mg/kg CP55940 and 3.0 mg/kg SR141716A. **B** Dose (mg/kg) of morphine administered in combination with 1.0, 3.2 and 10.0 mg/kg URB597. **C** Dose (mg/kg) of morphine administered in combination with 3.2, 5.6, 10.0 and 17.0 mg/kg AM404

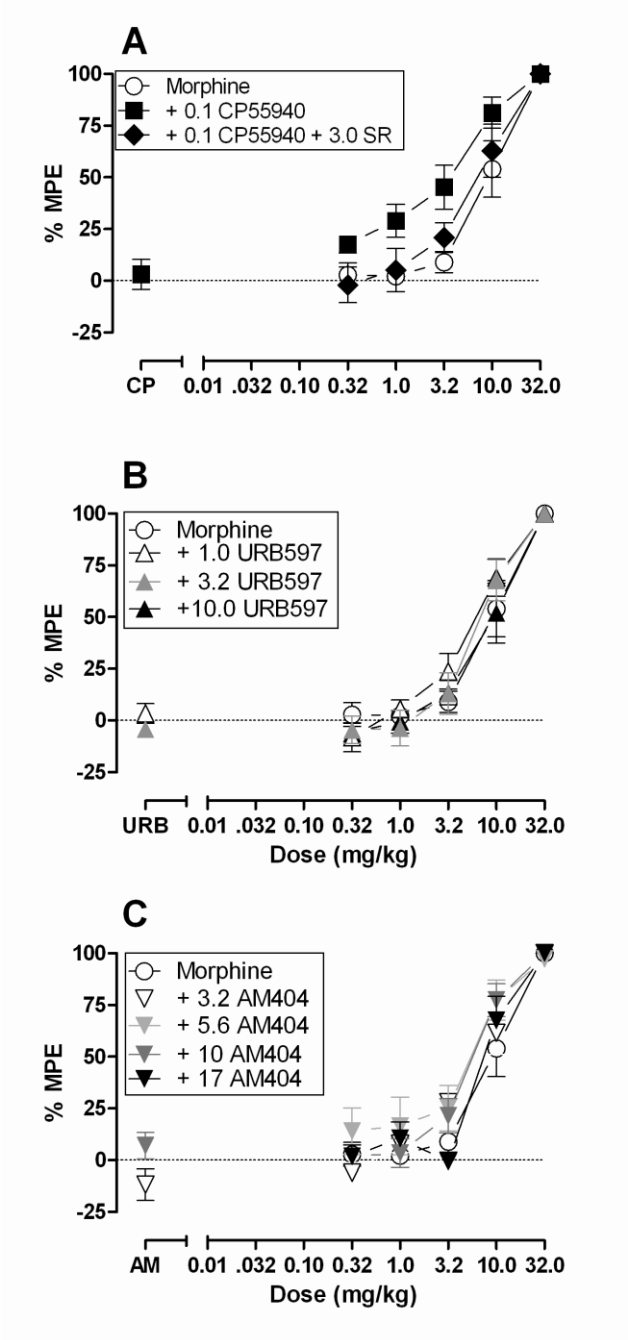


Fig. 3.3 Effects of CP55940, URB597, AM404 and morphine in the writhing assay. Ordinate: % inhibition of writhing. Abscissa: **A** dose (mg/kg) of each drug administered alone. **B** Dose (mg/kg) of CP55940 administered in combination with SR141716A. **C** Dose (mg/kg) of URB597 administered in combination with SR141716A. **D** Dose (mg/kg) of AM404 administered in combination with SR141716A

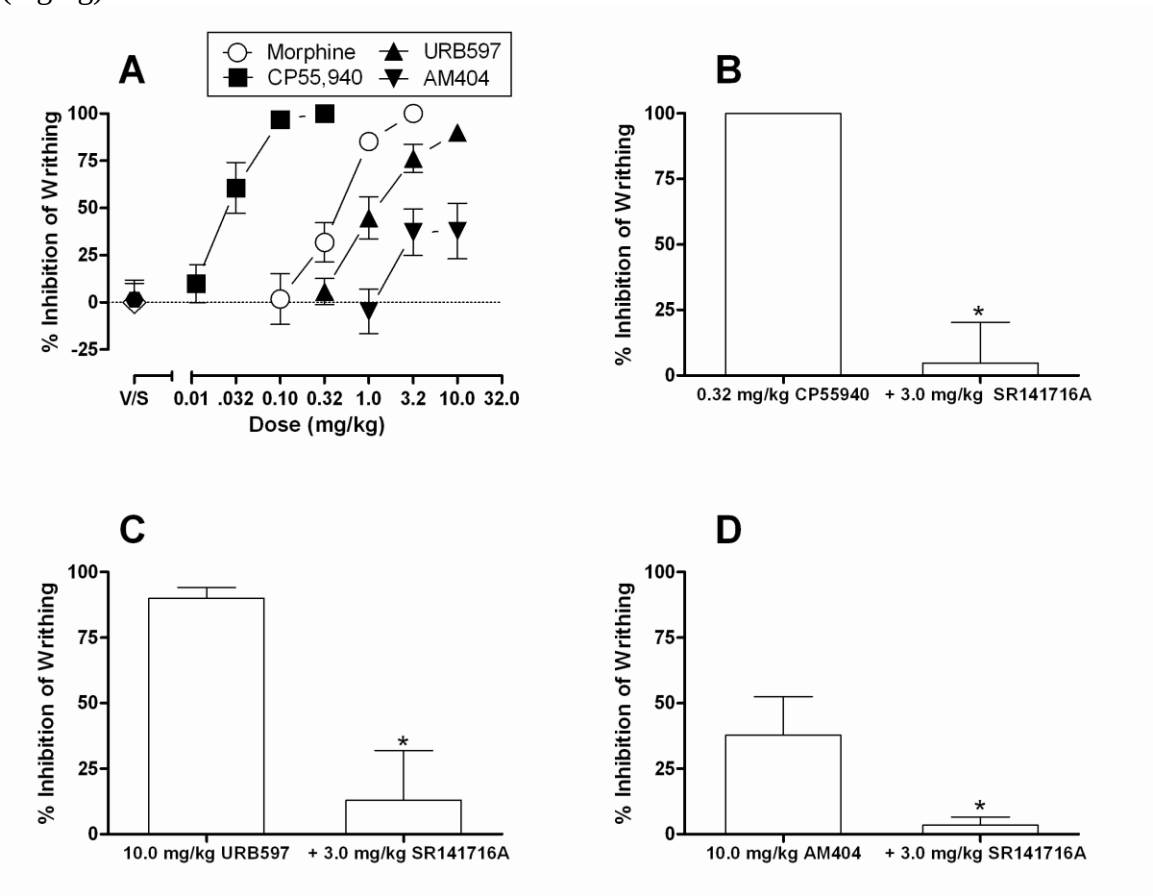
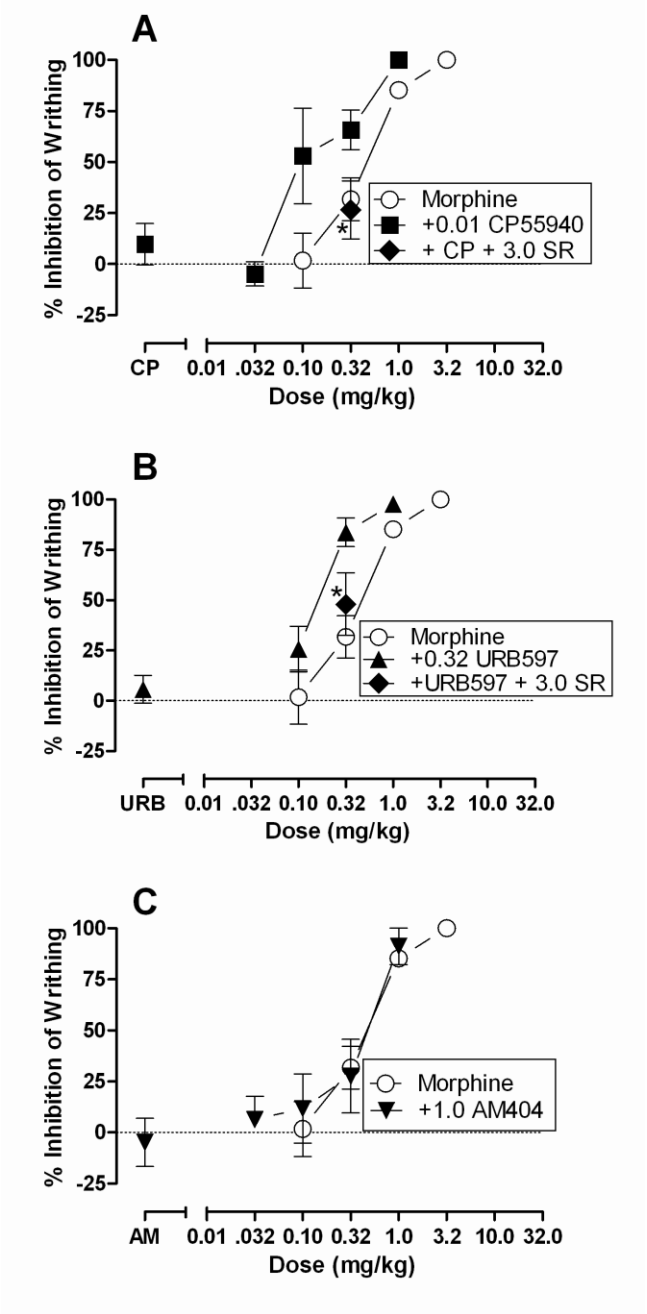


Fig. 3.4 Effects of morphine alone and in combination with CP55940, URB597 and AM404 in the writhing assay. Ordinate: % inhibition of writhing. Abscissa: **A** Dose (mg/kg) of morphine administered in combination with 0.01 mg/kg CP55940 and 3.0 mg/kg SR141716A. **B** Dose (mg/kg) of morphine administered in combination with 0.32 mg/kg URB597. **C** Dose (mg/kg) of morphine administered in combination with 1.0 mg/kg AM404. Asterisk indicates that the addition of SR141716A results in a significant difference from morphine + CP55940 or morphine + URB597



CHAPTER 4

EXPERIMENT 3: Behavioral effects of anandamide modulation and interactions with morphine: pain-suppressed and schedule-controlled behavior

ABSTRACT

Rationale Agonists at type 1 cannabinoid receptors produce antinociception and other effects including disruption of locomotor activity, catalepsy, and disruption of thermoregulation. Drugs that inhibit the degradation of the endogenous cannabinoid ligand anandamide, produce antinociception but typically do not produce other effects associated with CB1 agonists. It has been shown that CB1 agonists and anandamide modifiers enhance the antinociceptive effects of morphine. There is also evidence that CB1 agonists alter opioid effects on behavioral endpoints unrelated to nociception, but to date, the limited data available suggests that anandamide modifiers selectively alter morphine antinociception.

Objectives To determine if pharmacological inhibition of AEA degradation specifically results in antinociception, the effects of URB597 and AM404 were examined in two assays of pain-suppressed behavior (acetic acid-suppressed feeding and wheel-running) and an assay of schedule-controlled behavior. These assays were used to examine URB597 and AM404 in combination with morphine in order to determine if AEA modulates the effects of morphine on behaviors other than pain-elicited behaviors. The

effects of the CB1 agonist, CP55940 were determined in order to examine the specificity of the interactions between morphine and a direct agonist at CB1 receptors.

Results URB597, AM404 and morphine attenuated the suppression of wheel-running that resulted from administration of acetic acid; however only URB597 was effective at blocking the suppression of feeding. CP55940 was not effective in the assays of pain-suppressed behavior. URB597 was the only drug to enhance the effects of morphine in either of these assays. The CB1 agonist CP55940 and morphine dose-dependently decreased responding in the assay of schedule-controlled behavior, but URB597 and AM404 did not alter response rates. In addition, CP55940 but not URB597 or AM404 significantly enhanced morphine's rate-decreasing effects.

Conclusions These data are consistent with findings suggesting that modification of AEA availability, particularly through FAAH inhibition, produces antinociception without nonspecific effects that are associated with CB1 agonists. In addition, these findings provide evidence that AEA specifically modulates morphine's antinociceptive effects. On the other hand, these studies suggest that a direct CB1 agonist enhances morphine's effects on behavioral endpoints unrelated to antinociception.

INTRODUCTION

Recent research reveals that enhancement of endogenous cannabinoid signaling can produce antinociception. Pharmacological manipulations (Lichtman et al., 2004; Costa et al., 2006, 2010) that inhibit the activity of FAAH or inhibit AEA uptake result in increased levels of AEA that are accompanied by type 1 cannabinoid (CB1) receptor-dependent antinociception. To date, most evidence suggests that this effect is typically not accompanied by catalepsy, hypothermia, or changes in feeding or locomotor activity (Kathuria et al., 2003; Jayamanne et al., 2006; Clapper et al., 2010), which are other behavioral effects that are associated with the administration of CB1 agonists.

The results of several studies indicate that endogenous cannabinoids modulate the antinociceptive effects of opioids. Exogenously administered AEA, in combination with the FAAH inhibitor URB597 (Haller et al., 2008) enhances the antinociceptive effects of morphine. In addition, methylarachidonoylflurophosphate (MAFP), which inhibits the degradation of the endogenous cannabinoids AEA and 2-arachidonoylethanolamine (2-AG), enhances the antinociceptive effects of morphine (Pacheco et al., 2008, 2009) as well. Our laboratory has also shown (Experiment 2) that URB597 enhances the antinociceptive effects of morphine in the acetic acid-induced writhing assay, though there was no enhancement of morphine's antinociceptive effects in the thermal hotplate assay.

Consistent with the emerging data regarding manipulations of endogenous cannabinoid signaling, it is well-established that CB1 and opioid receptor agonists interact in assays of antinociception. For instance, exogenous cannabinoid agonists such

as delta-9-tetrahydrocannabinol (Δ9-THC) potentiate the effects of morphine in the mouse tail-flick test (Cichewicz and McCarthy, 2003) and the rat paw pressure test (Cox et al., 2007). However, there is also evidence suggesting that CB1 agonists alter the effects of opioids on other endpoints. For instance, CB1 agonists enhance morphine conditioned place preference (Manzanedo et al., 2004), the reinforcing efficacy of heroin in a self-administration paradigm (Solinas et al., 2005), and morphine's effects on locomotor activity (Ayhan et al., 1979).

It is well known that drug effects are determined by the behavioral endpoint being examined (Stevenson et al., 2003, 2005; Fischer and Dykstra, 2006; Fischer et al., 2008b), but as mentioned above, there is evidence that direct CB1 agonists enhance opioid effects across a variety of behavioral endpoints. Fewer studies have examined the effects of modification of AEA levels on the behavioral effects of morphine, but the FAAH inhibitor URB597 and the AEA uptake inhibitor AM404 do not alter the reinforcing effects of heroin (Solinas et al., 2005). Therefore, it may be the case that drugs that modify AEA, may enhance morphine antinociception without enhancing some of morphine's non-antinociceptive effects.

Recently researchers have demonstrated that preclinical pain models that examine pain-suppressed behavior may be useful in separating antinociceptive effects of drugs from non-specific effects such as disruptions of locomotor activity (Stevenson et al., 2006, 2009). The latter effects are a potential source of confounds in traditional pain models that utilize pain-elicited behavior. In addition, since antinociception and effects on locomotor activity are consequences of the administration of CB1 agonists and

morphine, assays of pain-suppressed behavior might be particularly helpful in separating antinociception from non-specific effects.

Accordingly, the present study has two goals. First, in order to extend the findings from experiment 2, the effects of administration of a direct CB1 agonist, CP55940 and AEA modifiers, URB597 and AM404, were determined on a variety of behavioral endpoints. Dose-effect curves were determined for each drug in two assays of pain-suppressed behavior (pain-suppressed feeding and pain-suppressed wheel-running). In addition, an assay of food-maintained schedule-controlled behavior was used to determine the effects of these drugs on a behavioral endpoint unrelated to nociception. The role of CB1 receptors in the effects of these drugs was assessed by the administration of the CB1 antagonist, SR141716A. Second, based on the evidence that AEA modifiers enhance the antinociceptive effects of morphine, but not effects unrelated to antinociception, we tested the hypothesis that blockade of AEA degradation and uptake would enhance morphine's antinociceptive effects in the assays of pain-suppressed behavior, but would not alter morphine's rate-decreasing effects in the assay of schedule-controlled behavior.

METHODS

Subjects

Male C57BL/6 mice purchased from the Jackson Laboratory and bred in house were group housed in the animal facilities of the Department of Psychology at the University of North Carolina at Chapel Hill. Mice of approximately 12 weeks of age at the beginning of testing were used in these studies. Mice had free access to food and

water except for the 2.5 h prior to experimental sessions. Lights were programmed on a 12 h light/dark cycle with lights off at 7:00am. All experiments took place during the dark cycle. Animal protocols were approved by the institutional animal care and use committee, and the methods were in accord with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996)

Experimental Procedures

Acetic acid-suppressed feeding

Mice were group housed and had free access to food and water until 23 and 5 h prior to testing, respectively. Mice were habituated to the testing room and handling for two days prior to testing. In addition, 24 h prior to testing, mice were given a 30 m session of free access to approximately 14 ml of 32% liquid nutrition (Vanilla flavor, CVS brand; composition as used: protein = 0.36 g/oz, carbohydrate = 1.6 g/oz, and fat = 0.24 g/oz). During this session, individual mice were placed into bedding-free polycarbonate mouse cages that contained a glass dish containing liquid nutrition and were allowed to explore the testing environment and consume the liquid nutrition.

On the test day, separate groups of mice were injected with saline or morphine (0.1 – 3.2 mg/kg; 45 m prior to the session), CP 55940 (0.01-0.32; 1 h prior to the session), URB597 (0.32-10.0; 1 h prior to the session), and AM404 (1.0-10.0; 1 h prior to session). In experiments examining the cannabinoid compounds in combination with morphine, the pretreatment times above were maintained and ineffective doses of CP55940, URB597 and AM404 were administered prior to morphine. In experiments

examining the effects of SR141716A, 3.0 mg/kg was administered 75 m prior to testing. Immediately prior to the session, mice were injected with 0.56% acetic acid or saline in control experiments. Following the second injection mice were immediately placed into clean polycarbonate mouse cages (11.5" X 7.5" X 5"; Allentown Inc, Allentown, NJ) containing liquid nutrition for 30 m. Consumption was quantified by subtracting the weight of the liquid-containing dishes after the session from the weight obtained prior to the session. Because body size might influence consumption independent of other variables, this value was divided by the animals' weight (grams of liquid consumed per gram of body weight). In the event of spillage, the data were discarded. The effect of each treatment on pain-suppressed consumption is expressed as percent of non-suppressed consumption (% Control): [(acetic acid-suppressed consumption (g/g) / non-suppressed consumption (g/g)) X 100].

Acetic acid-suppressed wheel-running

Mice were grouped housed in standard polycarbonate mouse cages (11.5" X 7.5" X 5"; Allentown Inc, Allentown, NJ) until the start of the experiments. At this time mice were individually housed in polycarbonate cages (14" X 10.5" X 5.5"; Tecniplast USA Inc., Exton, PA) containing running wheels (ENV-044, Med Associates, St. Albans, VT). Testing occurred after two weeks of habituation to handling and acquisition of wheel-running behavior. On the day prior to testing (control session) mice were injected with saline followed 45 m later by a second injection of saline and wheel-running (revolutions) were recorded for 30 m. The next day (test session), 24 h after the control session, separate groups of mice were injected with morphine (0.32 – 3.2 mg/kg; 45 m

prior to the session), CP55940 (0.01-0.1; 1 h prior to the session), URB597 (0.32-10.0; 1 h prior to the session), AM404 (1.0-10.0; 1 h prior to session) or saline/vehicle. Immediately prior to the session, mice were injected with 0.56% acetic acid or saline, and wheel-running was recorded for 30 m. In experiments examining the cannabinoid compounds in combination with morphine, the pretreatment times above were maintained and ineffective doses of CP55940, URB597 and AM404 were administered prior to morphine. In experiments examining the effects of SR141716A, 3.0 mg/kg was administered 75 m prior to testing. Acetic acid suppressed wheel-running was examined up to four times in each mouse with at least one week between exposures to acetic acid. This schedule of exposure did not have effects on wheel-running beyond the test day and does not produce long term disruptions of other behaviors, such as feeding (Stevenson et al., 2006). The effect of each treatment on pain-suppressed wheel-running is expressed as percent of non-suppressed wheel-running (% Control): [(acetic acid-suppressed running / non-suppressed running) X 100].

Schedule-controlled behavior

Mice were group housed in polycarbonate mouse cages (11.5” X 7.5” X 5”; Allentown Inc, Allentown, NJ) and had free access to food and water except for the 2.5 hr preceding experimental sessions. Mice were habituated to the testing room and handling for two days prior to testing. Response rates in the assay of schedule controlled behavior were determined in mouse operant chambers (8.5" X 7.0" X 5.0"; ENV-307W-CT; Med Associates, St. Albens, VT) that were equipped with a grid floor, houselight, ventilator fan, and two nose-poke holes (capable of being illuminated from within)

located on either side of a liquid dipper. Stimulus lights located above each nose-poke hole were not used during these experiments.

During experimental sessions mice were placed into the test chambers for 10 m without any of the stimuli activated. Following this acclimation period, the ventilator fan was activated and the left nose-poke hole was illuminated. At this point, left nose-poke responses counted toward completion of a fixed ratio (FR) response requirement and nose-pokes on the right had no scheduled consequences. During an initial training period, the FR value was increased from 1 to 2 and then the terminal ratio of 4, which was used for the remainder of the experiments. Completion of the FR resulted in access to a liquid reinforcer (32% Liquid Nutrition, CVS) via the liquid dipper, and activation of the houselight for 8 s. In addition, the light within the left nose-poke hole was turned off during the 8 s reinforcer delivery period. Once the reinforcer delivery period elapsed, the dipper was lowered, the houselight was turned off, and the left nose-poke hole was illuminated signaling the next response period. Testing occurred 5 days per week and sessions ended after 30 m elapsed or when 100 reinforcers were earned.

Once response rates were stable under the FR 4 ratio of reinforcement, the effects of pharmacological manipulations were determined. Separate groups of mice were used to determine the effects of morphine (0.32 – 3.2 mg/kg), CP 55940 (0.01-0.1 mg/kg), and URB597 (0.32-17.0 mg/kg). Following the determination of the effects of these compounds, the mice were given a period of 2 weeks without receiving drugs before being redistributed into separate groups to determine the effects of AM404 (1.0-17.0 mg/kg) and drug combinations. Drug effects were assessed twice a week on Tuesdays

and Fridays. Drug data are expressed as a percentage of control response rates (% Control): [(responses per minute on test day / responses per minute on control day) X 100].

Drugs

Morphine sulphate, URB597 and SR 141716A were provided by the National Institute on Drug Abuse (Bethesda, MD, USA). AM404 was purchased from Tocris (Ellisville, MO), and CP 55940 was purchased from Sigma Aldrich (St. Louis, MO). Morphine (45 m pretreatment), CP55940 (1 h pretreatment), URB597 (1 h pretreatment), AM404 (1 h pretreatment), and SR141715A (75 m pretreatment) were injected subcutaneously at a volume of 0.1 ml/10 g.

Data Analysis

Data are presented as mean (SEM) % control feeding, wheel-running, or responding (see above). One- and two-way ANOVAs were used to determine drug effects and post hoc comparisons were conducted using Fisher's protected least significant difference. All statistical analyses were conducted with an alpha level of significance set at $p < 0.05$.

RESULTS

Table 4.1 shows that intraperitoneal injection of 0.56% acetic acid resulted in a significant decrease in feeding behavior [$t(10) = 11.32$, $p < 0.05$] with mean consumption (±SEM) of 0.01 (0.003) g of fluid per gram of body weight for mice receiving an acetic acid injection versus 0.11 (0.01) g/g body weight in control animals. As shown in Fig.

4.1A, URB597 was the only drug that attenuated the suppression of feeding produced by injection of acetic acid [$F(4,35) = 2.24$, $p < 0.05$] and this effect was blocked by SR141716A (Fig. 4.1B).

Fig. 4.2 shows the effects of morphine in combination with ineffective doses of CP55940, URB597 and AM404. CP55940 (Fig. 4.2A) and AM404 (Fig. 4.2C) did not alter morphine's effects on pain-suppressed feeding. Fig. 4.2B shows, that though morphine had no effect on pain-suppressed feeding when administered alone, morphine in combination with an ineffective dose of URB597 (0.32 mg/kg) significantly attenuated the suppression of feeding [$F(3,22) = 49.15$, $p < 0.05$]. This effect was blocked by 3.0 mg/kg SR141716A.

Table 4.1 shows the effects of various drugs and drug combinations on non-suppressed feeding. When compared to control animals, 3.0 mg/kg SR141716A did not alter non-suppressed feeding under these experimental conditions. Likewise, 3.2 mg/kg URB597 and 0.32 mg/kg URB597 in combination with 0.32 mg/kg morphine, which were treatments that were effective at attenuating the suppression of feeding by acetic acid , did not alter non-suppressed feeding.

Table 4.2 shows that intraperitoneal injection of acetic acid also significantly suppressed wheel-running behavior [$t(14) = 4.04$, $p < 0.05$]. Mean (±SEM) wheel revolutions was 1101.75 (210.30) for control animals whereas wheel-running was completely suppressed in mice exposed to 0.56% acetic acid. Fig. 4.3A shows that URB597 [$F(4,35) = 4.03$, $p < 0.05$], morphine [$F(4,34) = 4.82$, $p < 0.05$] and AM404 [$F(3,28) = 6.82$, $p < 0.05$], but not CP55940 significantly attenuated the suppression of

wheel-running that resulted from i.p. injection of acetic acid. Fig. 4.3B and 4.3C shows that SR141716A significantly antagonized the effects of URB597 [$t(11) = 2.60$, $p < 0.05$] and AM404 [$t(14) = 2.55$, $p < 0.05$]. Doses of each drug that significantly attenuated the suppression of wheel-running by injection acetic acid were examined in mice in the absence of acetic acid, and each of these drugs had no effect on non-suppressed wheel-running (table 4.2).

CP55940 (Fig. 4.4A) and AM404 (Fig. 4.4C) did not alter morphine's effects in the wheel-running assay. Fig. 4.4B shows that 0.32 mg/kg URB597, which had no effect when administered alone, significantly altered the morphine dose effect curve in the wheel-running assay [$F(1,46) = 6.53$, $p < 0.05$]. Specifically, 0.56 mg/kg morphine in combination with 0.32 mg/kg URB produced a greater effect on wheel running than did this dose of morphine alone. SR1411716A antagonized this effect.

Fig. 4.5A shows the effects of morphine, CP55940, URB597, and AM404 in the assay of schedule-controlled behavior. ED50(95%CL) values were 4.36 (3.47-5.48) for morphine and 0.05 (0.05-0.06) for CP55940. URB597 and AM404 had no effect on responding. The rate-decreasing effect of CP55940 was antagonized by SR141716A (Fig. 4.5B).

Fig. 4.6A shows that an ineffective dose of CP55940 (0.032 mg/kg) significantly shifted the morphine dose-effect curve [ED50(95%CL) = 1.05 (0.81-1.45)] to the left yielding a potency ratio (95%CL) of 4.16 (3.01-5.75). Pretreatment with 3.0 mg/kg SR141616A, which had no effect on response rates when administered alone, attenuated the rate-decreasing effects of a combination of 0.032 mg/kg CP55940 and 3.2 mg/kg

morphine. URB597 (Fig. 4.6B) and AM404 (Fig. 4.6C) did not alter morphine's rate-decreasing effects.

DISCUSSION

The primary findings of this study were that the FAAH inhibitor, URB597 and the AEA uptake inhibitor, AM404, were effective to varying degrees in the assays of pain-suppressed behavior, but neither drug disrupted food-maintained schedule-controlled behavior. On the other hand, CP55940 was not effective in the assays of pain-suppressed behavior, and dose-dependently decreased response rates in the assay of schedule-controlled behavior. In addition, URB597 and AM404 did not enhance morphine's rate-decreasing effects in the assay of schedule controlled behavior, but URB597 significantly enhanced morphine's antinociceptive effects in the assays of pain-suppressed behavior. CP55940 enhanced morphine's rate-decreasing effects, but not morphine's antinociceptive effects.

Recent research has examined the consequences of pharmacological elevation of endogenous cannabinoid signaling on pain behaviors. Pharmacological manipulations that inhibit AEA degradation (Lichtman et al., 2004; Kinsey et al., 2009; Costa et al., 2010) or limit AEA uptake (Costa et al., 2006) are effective in preclinical pain models. Interestingly, the blockade of endocannabinoid degradation does not produce many of the non-antinociceptive effects that are associated with CB1 agonists, such as catalepsy and disruption of locomotor activity (Cravatt et al., 2001; Moore et al., 2005; Russo et al., 2007). This indicates that the behavioral endpoint used to determine the effects of these drugs is a determinant of their effects.

The results of the present experiments are consistent with these findings. First, the findings obtained from the assays of pain-suppressed behavior suggest that pharmacological inhibition of the breakdown of AEA, particularly FAAH inhibition by URB597 and to a lesser extent, inhibition of AEA uptake by AM404, produces antinociception that is not accompanied by effects that disrupt feeding or wheel-running behavior. In addition, doses of these drugs that reversed acetic-acid induced suppression of feeding and wheel-running did not alter these behaviors when administered in the absence of acetic acid. This suggests that the antinociception observed here (defined for these models as a return toward baseline rates of behavior) is not due to non-specific increases in the behaviors measured by these models. In contrast, a range of doses of URB597 and AM404, including doses that are effective in numerous pain models and produces changes in AEA levels (Fegley et al., 2004a, 2005), failed to alter response rates in the assay of schedule-controlled behavior. Taken together, these data suggest a behavioral selectivity of these drugs that CB1 agonists do not possess.

Previous studies have examined the interaction of the cannabinoid and opioid systems by administering CB1 agonists in combination with morphine, and have consistently shown that CB1 agonists enhance the antinociceptive effects of morphine (Smith and Martin, 1992; Welch and Stevens, 1992; Smith et al., 1994b; Welch et al., 1995; Massi et al., 2001). More recent studies utilizing CB1 antagonists provide evidence that endogenous cannabinoids are involved in the antinociceptive effects of morphine (Miller et al., under review; Pacheco et al., 2008, 2009). Moreover, morphine's antinociceptive effects are enhanced when the metabolism of AEA is inhibited (Pacheco et al., 2008, 2009; Haller et al., 2008).

The present studies demonstrate that URB597 enhanced morphine antinociception in the pain-suppressed feeding assay and in the pain-suppressed wheel-running assay in a CB1 receptor-mediated manner. However, URB597 did not alter morphine's effects on schedule-controlled behavior. These findings suggest that enhanced activity at CB1 receptors that results from inhibition of AEA degradation specifically enhances morphine's antinociceptive effects. On the other hand, the CB1 agonist CP55940 enhanced morphine's rate-decreasing effects in the assay of schedule-controlled behavior. Together with the findings in Experiment 2, these data suggest that behavioral endpoint is a determinant of the interaction between morphine and drugs that modify endogenous cannabinoid levels, whereas CB1 agonist enhance the effects of morphine across a variety of endpoints.

The mechanisms of this selectivity warrants further research, but there is evidence that tonic exposure to noxious stimuli results in elevations of AEA levels in regions associated with pain processing (Mitrirattanakul et al., 2006; Agarwal et al., 2007). Therefore, it may be the case that the effects of drugs that alter AEA levels are amplified in areas that have implications on pain signals. Consistent with this hypothesis, there is recent evidence that a peripherally acting FAAH inhibitor, URB937, produces CB1-dependent antinociception in a variety of pain models that involve tonic exposure to noxious stimuli, including acetic acid (Clapper et al., 2010). In addition, this peripheral FAAH inhibition produces CB1-dependent suppression of dorsal horn responses to formalin injection to the hind paw of rats (Clapper et al., 2010). Thus FAAH inhibition may magnify an endogenous response to noxious stimuli that includes increased AEA, and the resulting attenuation of pain related signaling might occur prior to the

involvement of the CNS. The effects of the drugs used in the present studies are not limited to the periphery, but the effects obtained in these studies are consistent with the possibility of a selective enhancement of AEA levels in areas involved in nociceptive processing as opposed to the wide-ranging activation of CB1 receptors resulting from CB1 agonists. This might explain the behavioral selectivity of the effects of AM404 and URB597, as well as the interactions of URB597 and morphine, that were observed here.

The present findings provide further evidence of the involvement of endogenous cannabinoids in the antinociceptive effects of morphine (Pacheco et al., 2008, 2009; Haller et al., 2008). It also appears that AEA involvement in the effects of morphine might be limited to antinociception, but thus far data regarding this hypothesis are limited. Further studies using different noxious stimuli, measures of antinociception, and behavioral endpoints are needed to fully characterize the role of endogenous cannabinoids in the behavioral effects of opioids.

Table 4.1 Liquid nutrition consumption by control mice, mice receiving 0.56% acetic acid in the absence of drug treatments, and mice receiving drug treatments in the absence of acetic acid. Asterisk indicates a significant difference from control animals

Acetic Acid Concentration	Drug Condition	Mean (SE) Consumption (g/g)
0	Saline	0.11 (0.01)
0.56%	Saline	0.01 (0.003)*
0	3.0 mg/kg SR1417116A	0.10 (0.01)
0	3.2 mg/kg URB597	0.09 (0.01)
0	0.32 mg/kg URB597 + 0.32 mg/kg morphine	0.09 (0.02)

Table 4.2 Wheel running by control mice, mice receiving 0.56% acetic acid in the absence of drug treatments, and mice receiving drug treatments in the absence of acetic acid. Asterisk indicates a significant difference from control animals

Acetic Acid Concentration	Drug Condition	Mean (SE) Revolutions
0	Saline	1101.75 (210.30)
0.56%	Saline	0.00 (0.00)*
0	3.0 mg/kg SR1417116A	775.38 (178.04)
0	1.0 mg/kg morphine	998.63 (210.30)
0	10.0 mg/kg URB597	945.17 (242.83)
0	10. mg/kg AM404	961.00 (266.01)
0	0.32 mg/kg URB597 + 0.56 mg/kg morphine	794.17 (104.56)

Fig. 4.1 Effects of CP55940, URB597, AM404 and morphine in the pain-suppressed feeding assay. Ordinate: % control feeding. Abscissa: **A** dose (mg/kg) of each drug administered alone. **B** Dose (mg/kg) of URB597 administered in combination with SR141716A

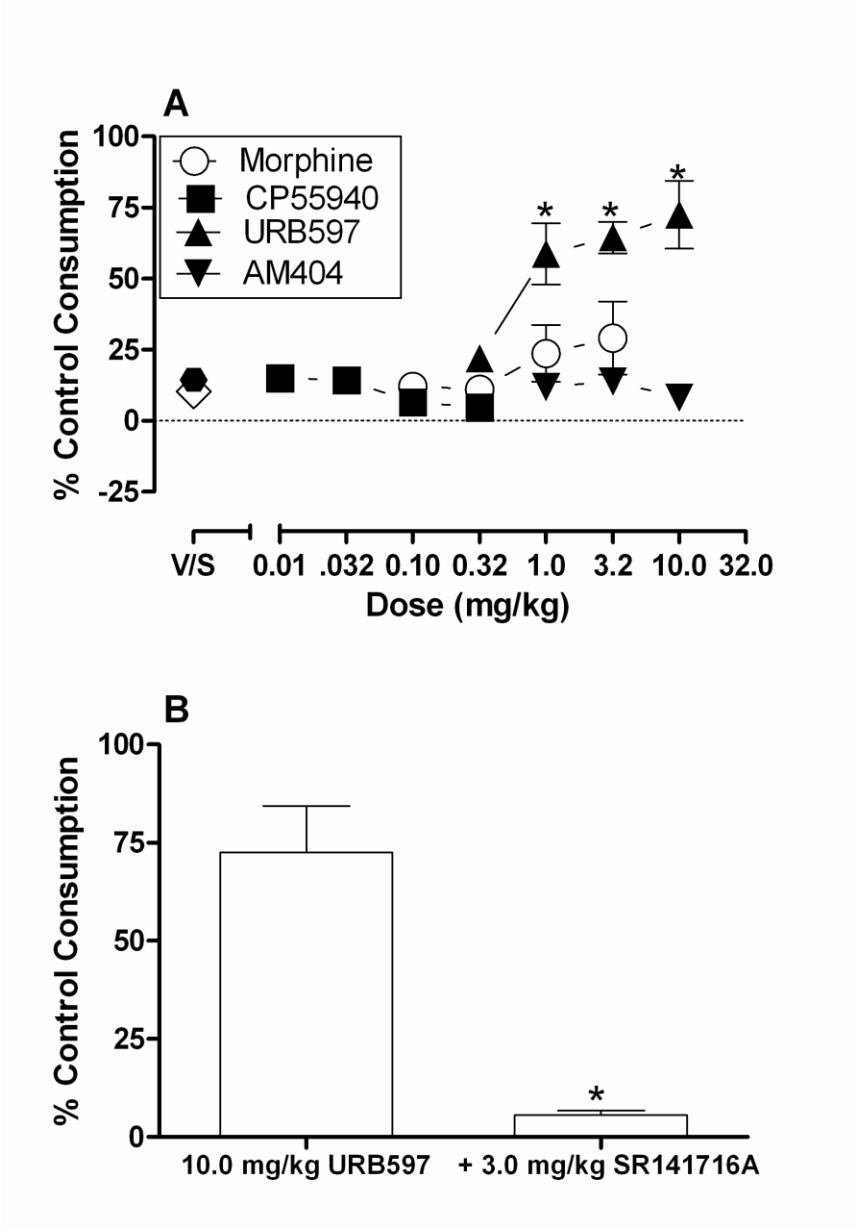


Fig. 4.2 Effects of morphine alone and in combination with CP55940, URB597 and AM404 in the pain-suppressed feeding assay. Ordinate: % control consumption. Abscissa: **A** Dose (mg/kg) of morphine administered in combination with 0.01 and 0.032 mg/kg CP55940. **B** Dose (mg/kg) of morphine administered in combination with 0.32 mg/kg URB597 and 3.0 mg/kg SR141716A. **C** Dose (mg/kg) of morphine administered in combination with 1.0 and 3.2 mg/kg AM404. Asterisks indicate significant difference relative to vehicle treated mice

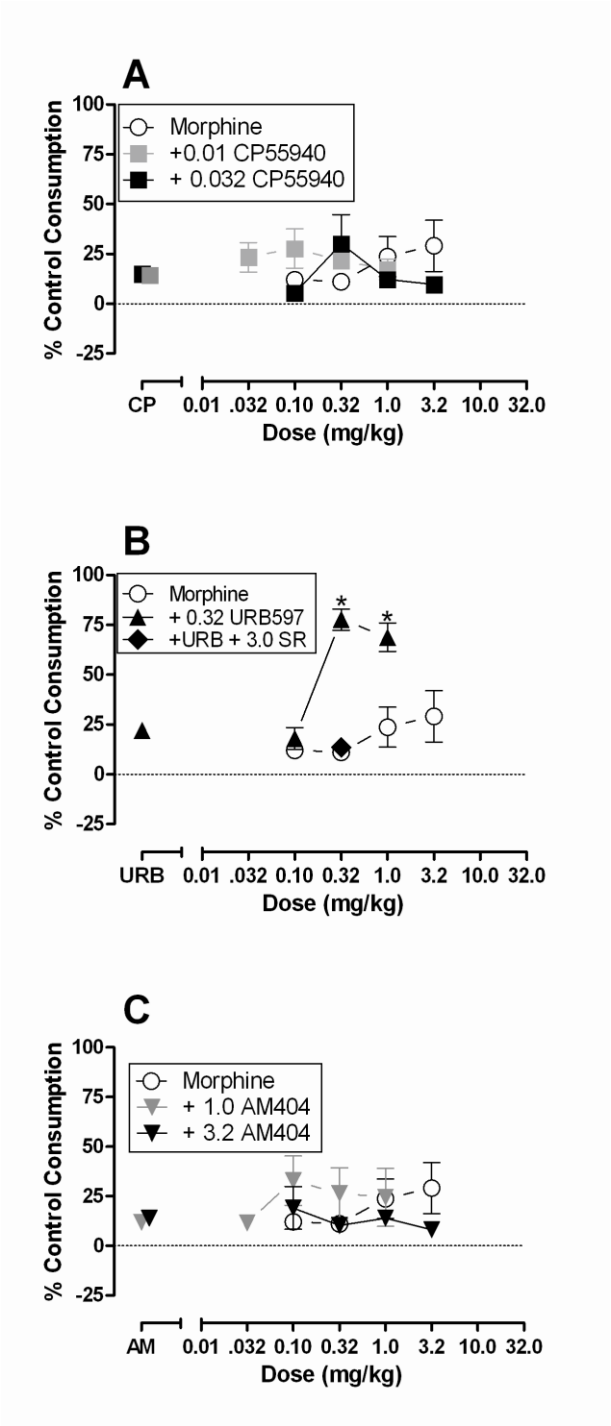


Fig. 4.3 Effects of CP55940, URB597, AM404 alone and in combination with morphine in the pain-suppressed wheel-running assay. Ordinate: % control running. Abscissa: **A** dose (mg/kg) of each drug administered alone. **B** Dose (mg/kg) of morphine administered in combination with 0.01 and 0.032 mg/kg CP55940. **C** Dose (mg/kg) of morphine administered in combination with 0.32 mg/kg URB597 and 3.0 mg/kg SR141716A. **D** Dose (mg/kg) of morphine administered in combination with 1.0 mg/kg AM404

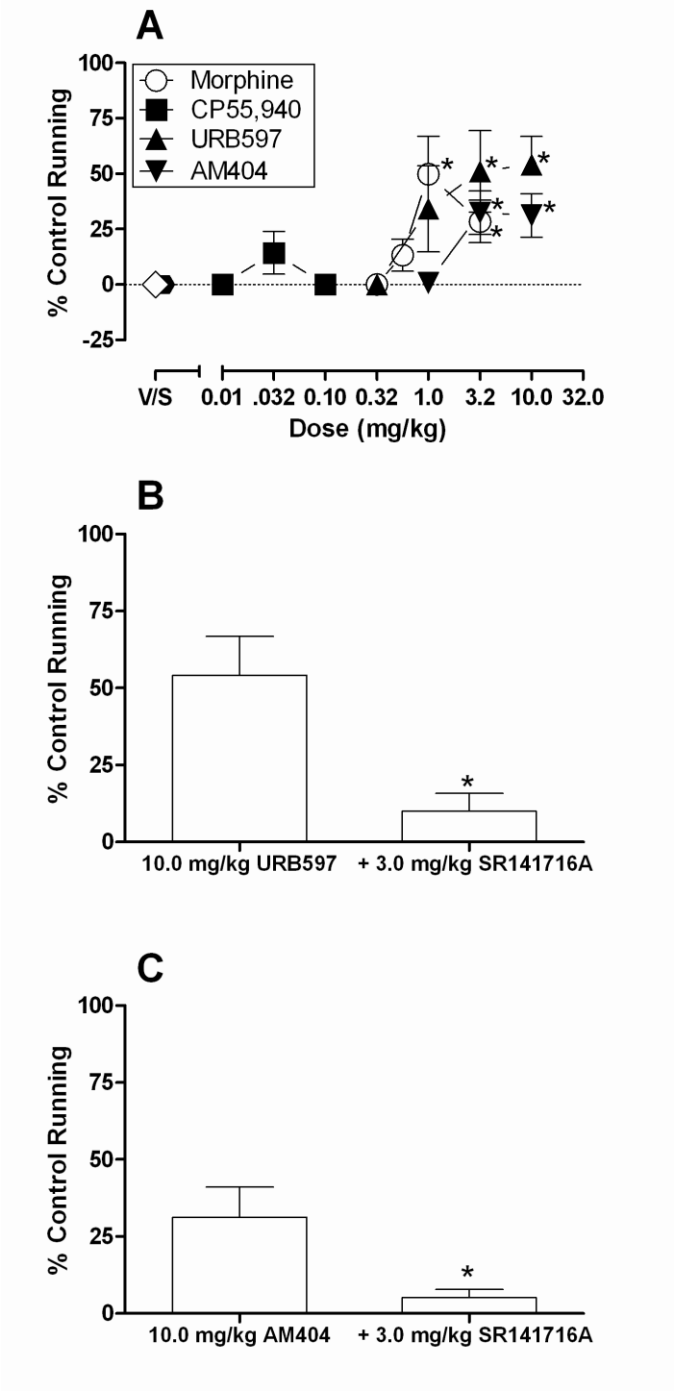


Fig. 4.4 Effects morphine alone and in combination with CP55940, URB597 and AM404 in the pain-suppressed wheel running assay. Ordinate: % control running. Abscissa: treatment. **A** Dose (mg/kg) of morphine in combination with 0.01 and 0.032 mg/kg CP55940. **B** Dose (mg/kg) of morphine in combination with 0.32 mg/kg URB597 and 3.0 mg/kg SR141716A. **C** Dose (mg/kg) of morphine in combination with 1.0 mg/kg AM404.

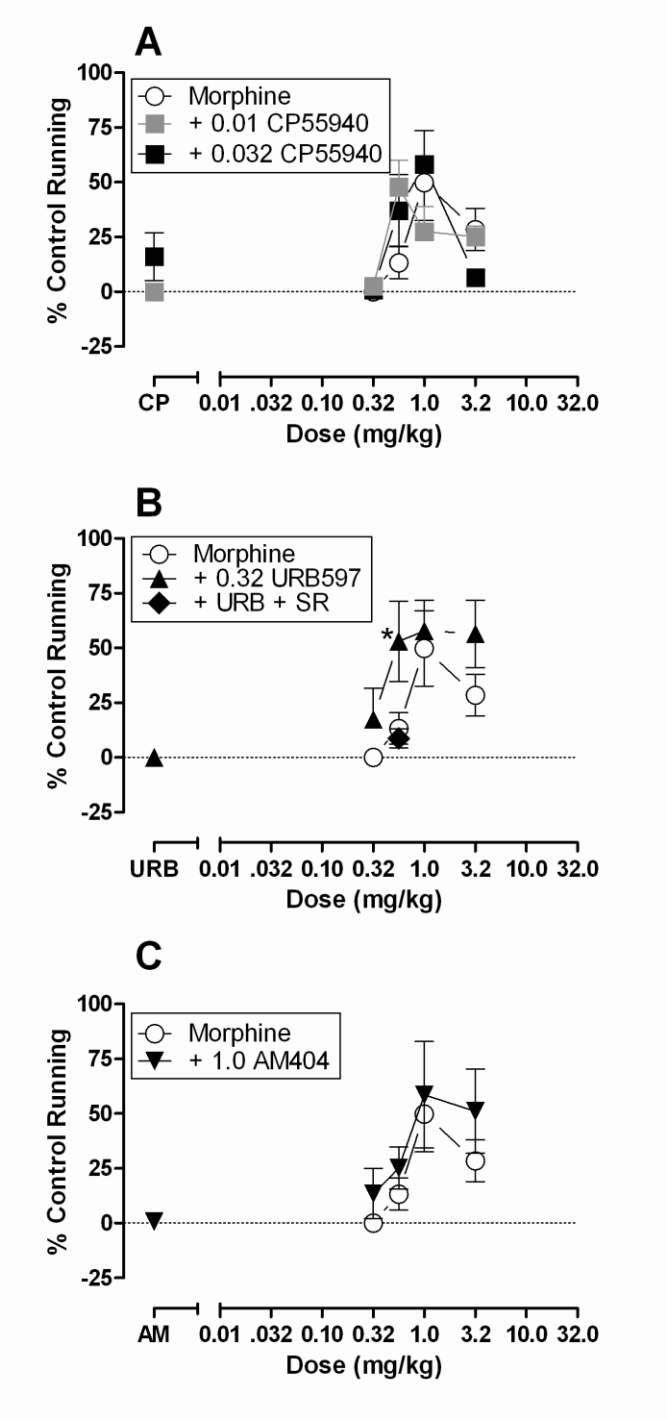


Fig. 4.5 Effects of CP55940, URB597, AM404 and morphine in the assay of schedule-controlled behavior. Ordinate: % control responding. Abscissa: **A** dose (mg/kg) of each drug administered alone. **B** Dose (mg/kg) of CP55940 administered in combination with SR141716A.

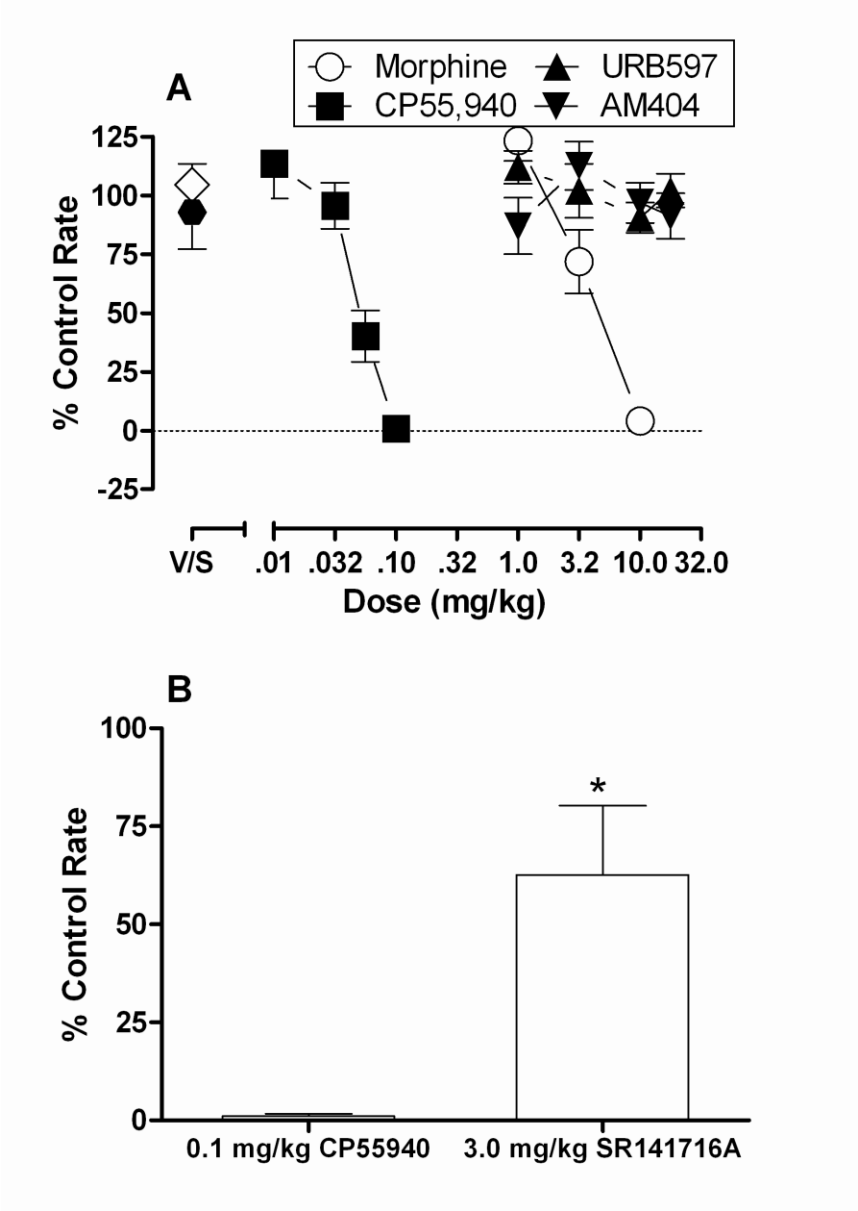
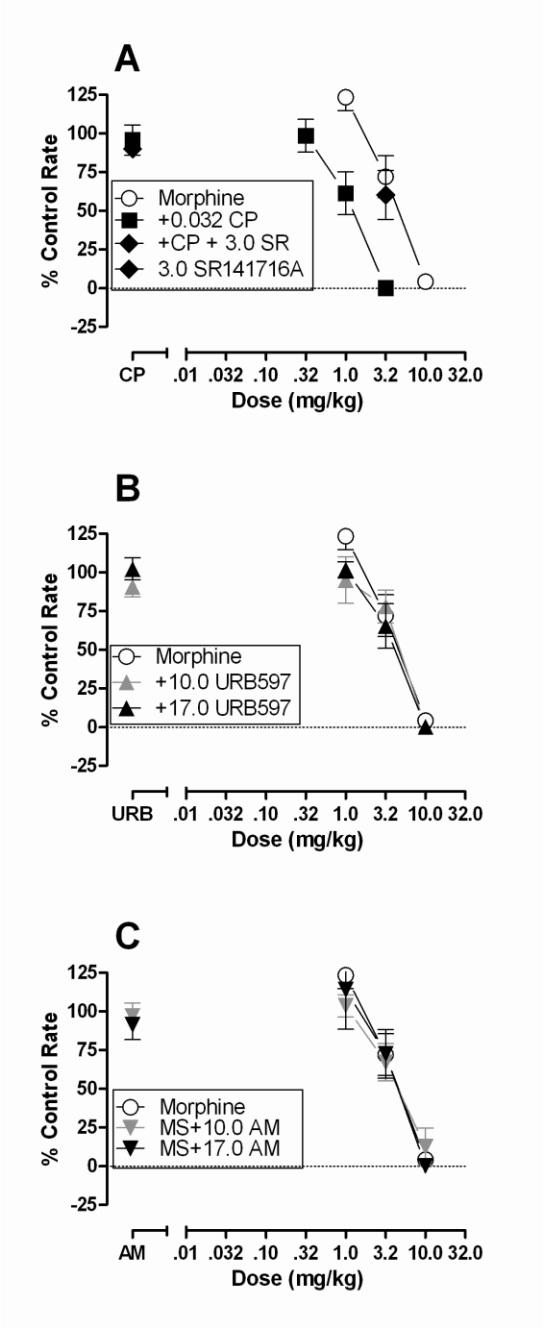


Fig. 4.6 Effects of morphine alone and in combination with CP55940, URB597 and AM404 in the assay of schedule-controlled behavior. Ordinate: % control feeding. Abscissa: **A** Dose (mg/kg) of morphine in combination with 0.032 CP55940 and 3.0 mg/kg SR141716A. **B** Dose (mg/kg) of morphine in combination with 10.0 and 17.0 mg/kg URB597. **C** Dose (mg/kg) of morphine in combination with 10.0 and 17.0 mg/kg AM404



CHAPTER 5
GENERAL DISCUSSION

Experimental Results

The experiments included in this project were designed with two goals in mind. The primary goal was to examine the role of endogenous cannabinoid signaling in the antinociceptive and other behavioral effects of morphine. A secondary goal was to assess the utility of models of pain-suppressed behavior, a relatively novel way of studying nociception and antinociception.

The first experiment used the strategy of disrupting cannabinoid signaling in order to determine the role of endogenous cannabinoids in morphine's antinociceptive effects. Previous studies had utilized CB1 knockout mice and selective CB1 antagonists to study the antinociceptive effects of morphine and obtained conflicting results. Studies using CB1 antagonists implicated the CB1 receptor in morphine's antinociceptive effects, whereas studies using knockout mice revealed no differences between wildtype mice and mice lacking the CB1 receptor. Procedural differences made interpretation of these findings difficult, particularly since the use of CB1 knockout mice to study morphine antinociception was limited to thermal pain assays. Experiment 1 examined the effects of morphine in CB1 knockout and wildtype mice in preclinical pain models that used both thermal and chemical noxious stimuli and measured a variety of behavioral responses to these stimuli. In addition, to determine whether CB1 knockout mice undergo developmental alterations that mask the role of this receptor in morphine's

antinociceptive effects, experiments were carried out with the selective CB1 antagonist, SR141716A.

The results from experiment 1 indicate that acute disruption of CB1 activity via pharmacological means does alter morphine's antinociceptive effects. In each instance in which morphine was efficacious, SR141716A attenuated its effect. In contrast with the pharmacological manipulation of CB1 receptors, but consistent with findings reported in the literature, knockout of the CB1 receptor had no consequences on morphine's antinociceptive effects. Together with the existing research, these data suggest that endogenous cannabinoids acting at CB1 receptors do have a role in the antinociceptive effects of morphine. On the other hand, we do not have a definitive explanation for the results obtained in knockout mice, but it is possible that these animals develop in a manner that circumvents the relationship between the cannabinoid and opioid systems, at least with regard to pain.

Experiment 2 used a different strategy to examine the role of endogenous cannabinoids in the antinociceptive effects of morphine. Recently, drugs that limit the degradation of anandamide and other endogenous cannabinoids have been synthesized and are being used to examine the effects of enhancing anandamide activity by preventing its enzymatic breakdown. We used the FAAH inhibitor, URB597 and the AEA uptake inhibitor, AM404, to determine whether pharmacological enhancement of AEA activity produces a similar profile of antinociceptive effects as the CB1 agonist CP55940. In addition, we sought to examine the role of AEA in the antinociceptive effects of morphine by determining the effects of URB597 and AM404 in combination with morphine.

Experiment 2 revealed that the antinociceptive effects of the FAAH inhibitor, URB597 and AEA uptake inhibitor, AM404, were dependent on the assay in which they were studied. Neither URB597 nor AM404 was effective in the hotplate assay, but both were effective to varying degrees in the writhing assay. Previous findings are mixed with regard to the efficacy of drugs that increase AEA levels in thermal pain assays, but the majority of findings suggest that these drugs produce antinociception in pain models involving inflammatory noxious stimuli (de Lago et al., 2002; La Rana et al., 2006).

In addition, experiment 2 provides support for the hypothesis that AEA modulates the antinociceptive effects of morphine; however this role may be dependent on the assay as well as the manner in which AEA levels are manipulated. URB597 enhanced the antinociceptive effects of morphine in the writhing assay, but neither URB597 nor AM404 altered morphine's effects in the hotplate assay. The CB1 agonist, CP55940 enhanced morphine's antinociceptive effects in both of these assays.

In experiment 3, two assays of pain-suppressed behavior and an assay of schedule-controlled behavior were used to extend the findings from experiment 2 with regard to antinociception, and to provide a behavioral endpoint unrelated to antinociception for examining these drugs. Consistent with our hypotheses and what is known about these drugs, URB597 and AM404 were effective in the assays of pain-suppressed behavior to varying degrees and did not alter response rates in the assay of schedule-controlled behavior. On the other hand, the CB1 agonist, CP55940 dose-dependently decreased response rates and had no effect on pain-suppressed behavior.

As expected, CP55940 resulted in a significant leftward shift of the morphine dose-effect curve in the assay of schedule-controlled behavior, but not the assays of pain-

suppressed behavior. AM404 did not alter the effects of morphine in any of the models used in this project. On the other hand, URB597 produced upward shifts in the morphine dose-effect curve in the pain-suppressed feeding and pain-suppressed wheel-running assays (indicative of enhancement), and did not alter the rate-decreasing effects of morphine in the assay of schedule-controlled behavior.

Taken together, experiments 2 and 3 suggest that unlike CP55940, URB597 selectively enhances morphine antinociception (in models employing chemical noxious stimuli) without enhancing nonspecific effects such as disruption of locomotor activity. These findings are consistent with data suggesting a specific role of AEA in the antinociceptive effects of morphine.

Disparities in the effects of URB597 and AM404

There is mounting evidence that the endogenous cannabinoid system, specifically the inhibition of the metabolism of endogenous cannabinoids, is a potential target for the treatment of pain. Among the strategies receiving attention is the pharmacological inhibition of FAAH. Administration of drugs that block the activity of this enzyme results in rapid and prolonged increases in anandamide levels (Lichtman et al., 2004; Fegley et al., 2005) and produces antinociception in a variety of preclinical models including the hotplate, tail-immersion and formalin tests (Lichtman et al., 2004) as well as neuropathic pain models (Kinsey et al., 2009).

While FAAH inhibitors disrupt the functioning of the enzyme that is primarily responsible for anandamide degradation, prevention of anandamide uptake is another strategy that may be employed to study this system. There is some controversy about the

mechanism of AEA uptake (i.e. active transport or a passive process), but drugs that have been termed anandamide uptake inhibitors, such as AM404, result in increased anandamide levels (Giuffrida et al., 2000), and antinociception has been demonstrated with AEA uptake inhibitors in the formalin test (Gühring et al., 2002; La Rana et al., 2006), the chronic constriction injury and complete freund's adjuvant tests (La Rana et al., 2006). On the other hand, the data are mixed with regard to thermal tests (Beltramo et al., 1997, 2000; de Lago et al., 2002; Ruggieri et al., 2008).

Existing behavioral and physiological data suggest that URB597 and AM404 would have similar effects in the assays used here; however, that was not always the case. When both drugs were active in an assay and their effects could be compared directly, URB597 was typically more potent and efficacious than AM404. It is not entirely surprising that two drugs that alter anandamide levels via different means would produce different results. However, given the similar magnitude and time course of effects of these drugs on anandamide levels (Fegley et al., 2004a, 2005), and our hypothesis that it is anandamide activity at CB1 receptors that is mediating the behavioral effects of these drugs, this finding was not predicted.

The fact that AM404 is very similar in structure to anandamide (Beltramo et al., 1997) might play a role in these differences. AM404 is also similar to anandamide in that both bind to CB1 receptors, though AM404 binds with lower affinity (Khanolkar et al., 1996), and this binding is not accompanied by activation of CB1 receptors (Beltramo et al., 1997). In contrast, URB597 does not bind to CB1 receptors (Piomelli et al., 2006). Since AM404 enhances anandamide levels while binding to, but not activating CB1 receptor, this leaves the possibility that AM404 is inhibiting the uptake of anandamide

while simultaneously functioning as a low affinity competitive antagonist at CB1 receptors. To our knowledge, there are no studies that directly examine this possibility and this remains speculation.

Another possible explanation for the decreased efficacy of AM404 relative to URB597 is based in another similarity between AM404 and anandamide. There is evidence that AM404 serves as a substrate for FAAH (Fegley et al., 2004b). Breakdown of AM404 by FAAH would presumably inhibit the drug's ability to disrupt anandamide uptake. On the other hand, it seems that this would result in competition with anandamide for hydrolysis by FAAH (Di Marzo, 2006), perhaps compensating for the loss of AM404 through its role as a substrate for the enzyme. This is again only speculation, but provides rationale for studies examining the consequence of combinations of a FAAH inhibitors and anandamide uptake inhibitors.

Disparities in the effects of CP55940 and modification of AEA

Unlike the differences observed between the effects of URB597 and AM404, it was not surprising that the CB1 agonist produced a different profile of effects than URB597 and AM404. In fact, the prediction that this would be the case contributed to the rationale for Experiments 2 and 3. CP55940 is a full agonist at CB1 receptors (Pertwee, 1999), and our findings using the hotplate, writhing, pain-suppressed feeding and wheel running, and schedule controlled behavior assays are consistent with the known effects of CB1 agonists. In addition, although we did not quantify this effect here, we can report that we have seen dose-dependent disruption of locomotor activity after administration of CP55940 which is also a well-established effect of CB1 agonists.

As discussed previously, CB1 agonists activate presynaptic CB1 receptors, resulting in decreased synaptic activity. When administered systemically, CB1 agonists accomplish this throughout the nervous system. CB1 receptors are widely distributed in areas that are consistent with the behavioral effects of CB1 agonists. For instance, CB1 receptors are highly expressed in brain areas related to motor control such as the basal ganglia and cerebellum (Herkenham et al., 1991). Likewise, CB1 receptors are elevated in the periaqueductal gray, dorsal horn of the spinal cord, and other regions associated with pain processing (Herkenham et al., 1991). This is not an exhaustive representation of the widespread distribution of these receptors, but these regions and associated behaviors have direct relevance to the current studies. In the absence of efforts to selectively activate CB1 receptors in areas relevant to pain processing, it is not surprising that drugs acting as full agonists at CB1 receptors alter locomotor activity and produce other non-specific effects.

The relatively recent discovery of endogenous cannabinoids and ways to manipulate them has allowed for renewed targeting of the cannabinoid system for therapeutic goals, in addition to those of basic science. Disruption of AEA degradation by pharmacological means results in increased brain levels of AEA (Beltramo et al., 1997; Kathuria et al., 2003; Lichtman et al., 2004, 2008; Moore et al., 2005; Kinsey et al., 2009). Accordingly, this results in increased availability of this ligand for binding to CB1 receptors throughout the nervous system. On the other hand, there is evidence that in the absence of drugs, anandamide levels increase specifically in areas associated with pain transmission upon exposure to tonic noxious stimuli (Walker et al., 1999; Mitrirattanakul

et al., 2006; Jhaveri et al., 2007; Petrosino et al., 2007; Richardson et al., 2008; Kaufmann et al., 2009).

The mechanisms of noxious stimulus-induced increases in anandamide are unknown, and could be the result of increased synthesis or decreased metabolism of AEA. In any case, this presents a potentially parsimonious explanation for the apparent selectivity (i.e. antinociception but not other CB1 agonist-like effects) of drugs like URB597 and AM404. Though these drugs result in wide ranging increases in AEA levels, this effect may be amplified in areas relevant to pain processing because tonic exposure to noxious stimuli results in increased AEA in these areas. As a result, whereas systemically administered CB1 agonists have their effect wherever CB1 receptors are located, URB597 and AM404 would have an exaggerated effect in areas where AEA levels are already increased. There are limited studies examining this hypothesis but one study that examined the effects of URB597 on AEA levels in spinal nerve ligation (SNL) and sham-operated rats (Jhaveri et al., 2006) found that SNL-operated rats had higher levels of anandamide in their ipsilateral paw compared to sham-operated animals. This is indicative of noxious stimulus-induced increases in AEA. On the other hand, compared to sham-operated rats that received URB597 injections into the ipsilateral paw, SNL-operated rats that received the FAAH inhibitor had decreased AEA levels. In contrast to these findings, when URB597 was applied directly to the spinal cord of SNL-operated rats, they exhibited increased levels of AEA relative to sham-operated rats that received URB597. The mechanisms behind these disparate findings are unclear and this is an area that is the focus of ongoing research, but this study does suggest an interaction of FAAH inhibition and pain state on AEA levels.

Anandamide's activity as a partial agonist at CB1 receptors might also account for the fact that drugs such as URB597 and AM404 do not produce effects such as disruptions of locomotor activity or catalepsy. It is noteworthy that though URB597 was effective in three of the four pain assays utilized here (acetic acid-induced writhing, pain-suppressed feeding, and pain-suppressed wheel running), all of these assays rely on the same noxious stimulus (i.p. injection of acetic acid). The acetic acid writhing model is known to be relatively sensitive to analgesics and on their own, the present findings might be indicative of this. For instance, all of the drugs tested were effective, to some degree, at inhibiting the writhing response. When it was possible to compare the ED50 of a drug in the writhing assay to the ED50 for that drug in another model, all of the drugs were more potent at inhibiting writhing than affecting behavior in the other models. This is not direct support for the hypothesis that anandamide's partial agonist profile is why drugs like URB597 and AM404 do not cause the full array of CB1 agonist-like effects, but is consistent with some existing evidence. Other researchers have shown that partial and full exogenous agonists (as defined in vitro) at CB1 receptors share some behavioral effects (antinociception), differ in efficacy and potency on other endpoints (effects on thermoregulation), and can be differentiated according to the presence or absence of other effects (diuretic effects; Paronis et al., 2009).

The differing effects of CP55940, URB597 and AM404 in our studies might also indicate the importance of the type of the noxious stimulus in determining the antinociceptive effects of drugs and drug combinations. The thermal noxious stimulus used in the hotplate assay and the chemical noxious stimulus used in the writhing, pain-suppressed feeding, and pain-suppressed running assays differ with regard to stimulus

modality and duration (acute versus tonic). In addition, the hotplate and writhing assays rely on different sites of nociceptive processing; the hotplate assay relies on spinal and supraspinal processing (Morgan et al., 1989; Pastoriza et al., 1996) whereas there is recent evidence that peripheral anandamide mediates the response to acetic acid (Clapper et al., 2010). Any number and combination of these variables could contribute to differences between the effects of the cannabinoid agents.

Involvement of Endogenous Cannabinoids in the Antinociceptive Effects of Opioids

It is well established that CB1 agonists can enhance the effects of opioids and the results of the present studies with CP55940 are consistent with previous findings; however, the mechanisms by which this occurs are yet to be identified. Researchers have hypothesized that one factor in cannabinoid/opioid interactions is that exposure to cannabinoids and opioids results in reciprocal changes in receptor expression and/or function. Behavioral studies relevant to this hypothesis have yielded contradictory results. Studies have shown cross tolerance between opioids and cannabinoids (Thorat and Bhargava, 1994; Rowen et al., 1998; Massi et al., 2001), as well as cross sensitization (Rubino et al., 1997). These behavioral phenomena may indicate changes at the receptor level, but the differing results of these studies do not clarify the identity of the mechanisms involved in interactions between the cannabinoid and opioid systems.

Vigano et al. (2005) attempted to reconcile these differences by examining the antinociceptive effects of morphine and CP55940 in rats that had received chronic administration of CP55940 and morphine, respectively. In addition, they attempted to correlate their behavioral findings to changes at the receptor level. Chronic exposure to

morphine produced sensitization to the antinociceptive effects of CP55940, whereas chronic exposure to CP55940 resulted in cross tolerance to the antinociceptive effects of morphine. They also found that chronic exposure to morphine decreased CB1 density, but increased, or did not change (depending on brain region) CP55940 activity on cAMP. On the other hand, chronic exposure to CP55940 resulted in increased μ-opioid receptor density, and decreased DAMGO activity on cAMP. The findings of this study suggest that the interactions of cannabinoids and opioids are not necessarily symmetrical, but clearly demonstrate that cannabinoids and opioids can alter the amount and efficiency of receptors of the *other* system. These studies provide some interesting insights into the behavioral and physiological consequences of chronic exposure to cannabinoids and opioids, but all of the present studies used acute administration of the drugs, so the results obtained by Vigano et al. (2005) might not be entirely relevant to our findings.

Another possible mechanism behind cannabinoid/opioid interactions, which is not exclusive of the hypothesis above and may be more relevant to our studies, is that the administration of drugs that act on the cannabinoid and opioid systems alter the release and/or synthesis of endogenous ligands for receptors of the *other* system. Researchers studying the modulation of the cannabinoid system by endogenous opioids have obtained results that are consistent with this hypothesis. The cannabinoid agonist Δ9-tetrahydrocannabinol and morphine interact synergistically in assays of antinociception (Cichewicz and McCarthy, 2003; Cox et al., 2007), and kappa opioid receptor antagonists have been shown to attenuate the effects of cannabinoid agonists (Smith et al., 1994a; Pugh et al., 1995). Finally, it has been demonstrated that cannabinoid agonists increase dynorphin levels (Mason et al., 1999b, 1999a). Together, these findings provide strong

support for the hypothesis that antinociception produced by cannabinoid agonists is mediated by dynorphins.

Our studies provide indirect support for the hypothesis that endogenous cannabinoids are released in response to opioid administration, and thus mediate the antinociceptive effects of opioids. These experiments show that a selective CB1 antagonist attenuates the antinociceptive effects of morphine. In addition, using doses and pretreatment times that have been shown to produce significant elevations in anandamide levels (Fegley et al., 2005), we demonstrated that URB597 enhanced morphine antinociception in preclinical pain assays that utilize acetic acid as a noxious stimulus. Other recent studies have found similar findings. Pacheco et al. (2008, 2009) also saw attenuation and enhancement of morphine's antinociceptive effects by a selective CB1 antagonist and FAAH inhibitor, respectively. In addition, acute administration of morphine increases anandamide levels in rats (Vigano et al., 2004). Taken together, these results are consistent with the hypothesis that endogenous cannabinoids acting at CB1 receptors modulate the antinociceptive effects of morphine. Together with results of studies demonstrating interactions at the receptor and signal transduction level, these studies suggest a complex relationship between two biological systems that are of great importance and interest.

Preclinical Assessment of Antinociception

As stated previously, a secondary goal of this project was to examine the potential of preclinical pain models that measure pain-suppressed behavior relative to more traditional models of pain-elicited behavior. At a basic science level, it is widely accepted

that it is important to measure nociception and antinociception across a variety of endpoints, behavioral and otherwise, as this can be an important determinant of the efficacy and potency of an intervention. The development and evaluation of assays of pain-suppressed behavior is an extension of this thinking.

A more functional perspective argues for the potential of such models based on the fact that they might be less susceptible to certain confounds associated with more traditional models (e.g. disruption of the subject's ability to make the pain-elicited response). In addition, it could be argued that these models are more in line with what occurs at the clinical level. In humans (Melzack, 1975) and animals (Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, 1996), clinical assessment and treatment of pain is often based on the degree to which the pain state disrupts behaviors that would otherwise occur at a high rate.

Regarding the issue of confounds, it *is* likely that assays of pain-suppressed behavior are less susceptible to certain confounds associated with assays of pain-elicited behavior. If a drug specifically produces antinociception, it would allow pain-suppressed behaviors to return to baseline levels. On the other hand, if administration of a drug results in effects that inhibit the animals' ability to produce the measured response (e.g. disruption of locomotor activity), the pain suppressed behavior would not likely return to baseline levels. This is an advantage of such models; however, these models aren't without potential confounds of their own. In fact, the most obvious potential confound is basically the opposite phenomena. The effect of interest in models of pain-suppressed behavior is blockade of the suppression of behavior by pain (i.e. the ability of behavior to return to baseline levels). As a result, the researcher must determine if a return to baseline

is due to antinociception or drug-induced stimulation of the target behavior. For instance, in experiment 2, if URB597 significantly increased feeding behavior in the absence of a noxious stimulus, interpretation of its effects on pain-suppressed feeding behavior would be difficult. Thus, as would be expected, the use assays of pain-suppressed behavior does not eliminate the possibility of confounds or the need for careful experimental design and appropriate controls.

Another issue, with regard to the translational potential of assays of pain-suppressed behavior, is that it is not always the case that an increase in pain-suppressed behavior is a desirable or reasonably attainable outcome. Sometimes antinociception in the absence of a resumption of behavior is sufficient. In such situations the utility of models of pain-suppressed behavior would be greatly diminished.

A final consideration, from the perspective of the experimenter, has to do with the practicality of the studies. The advantages of assays of pain-elicited behavior such as the hotplate and tail-flick assays include 1) the fact that they require little to no training of the animal and do not require a great deal of time, 2) there are (typically) no special animal housing or care requirements, 3) a variety of noxious stimuli are easily administered, and 4) despite the statements above regarding the rationale for assays of pain-suppressed behavior, the confounds associated with these models are addressed fairly easily.

Regarding point 1, in the present studies, the pain-suppressed feeding assay did not require any training and in many ways (e.g. habituation to the testing room and apparatus, handling, etc) the procedure was similar to that that our laboratory uses for hotplate and tail-flick studies. Likewise, wheel-running requires no training, however, one obstacle that was initially encountered was that the injection procedure severely

disrupted running behavior (i.e. saline injections suppressed running) until mice were well-habituated to handling and injections. This does not take away from the validity of the results obtained here, but whereas the hotplate is a fairly high throughput behavioral model, the wheel-running assay, as designed here, requires far more time.

Many behavioral models (pain and otherwise) that use laboratory animals require special housing and care (point 2). Typically, assessments of the antinociceptive effects of drugs in assays of pain-elicited behavior do not warrant this; animals can be group housed and there is no need for special dietary considerations. The assays of pain-suppressed behavior used here each have one of these requirements. In the feeding model the mice were food deprived, and in the running model the mice were individually housed. As stated above, these requirements are not beyond those faced by many researchers, but compared to assays like the hotplate, they result in decreased efficiency.

Another advantage of traditional assays (point 3) is the ease with which a variety of noxious stimuli can be administered and thus examined. As discussed above, the nature (modality, duration, etc.) of noxious stimuli can be an important determinant of the antinociceptive effects of drugs. Therefore it is desirable for researchers to study drug effects across a range of nociceptive endpoints. Traditional assays of pain-elicited behavior accommodate this quite well. However, at this stage in their development, assays of pain suppressed behavior are only amenable to noxious stimuli that are tonically active.

Proponents of assays of pain-suppressed behavior often mention the confounds that are associated with assays of pain-elicited behavior when discussing the rationale for the former models (point 4). Although these arguments are valid, in reality, confounds

such as disruptions of locomotor activity can be addressed fairly easily. Assays such as the rotorod assay and assessments of locomotor activity require little training or special care, and are very informative about whether or not a potential analgesic compound is actually disrupting the animals ability to produce the measured response in assays of pain-elicited behavior.

These considerations are only meant to be an objective assessment of the potential utility of assays of pain-suppressed behavior. The results of the present studies are indicative of their usefulness in detecting antinociception in the absence of non-specific effects, but it would be very premature to consider them as a replacement for tradition preclinical pain assays. Nonetheless, it is important for researchers to continue to consider and develop new ways of examining nociception and antinociception.

Concluding Remarks

The experiments described herein detail efforts to contribute to the understanding of the role of the cannabinoid system in behavior, particularly in the behavioral effects of opioids. These studies reveal that acute disruption or enhancement of endogenous cannabinoid activity alters the antinociceptive effects of morphine, and that these effects are dependent on the behavioral endpoint by which they are examined. This dissertation expands on extensive ongoing research that continues to detail the many roles of the endogenous cannabinoid system.

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