INTERACTIONS BETWEEN OPIOID AGONISTS AND GLUTAMATE RECEPTOR ANTAGONISTS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology (Behavioral Neuroscience).

Chapel Hill
2008

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ABSTRACT

BRADFORD D. FISCHER: Interactions between Opioid Agonists and Glutamate Receptor Antagonists
(Under the direction of Linda A. Dykstra)

The following studies systematically examine the interactive effects of opioids and glutamate receptor antagonists in assays of schedule-controlled responding and thermal nociception.

Experiment 1 examines the effects of morphine, buprenorphine, butorphanol, and nalbuphine alone and in combination with an *N*-Methyl-D-Aspartate (NMDA) receptor antagonist on schedule-controlled responding and thermal nociception. The results from this study indicate that NMDA antagonist/morphine and NMDA antagonist/buprenorphine mixtures produce additive or infra-additive effects on schedule-controlled responding, whereas NMDA antagonist/butorphanol and NMDA antagonist/nalbuphine mixtures produced additive or supra-additive effects. In addition, mixtures of an NMDA antagonist in combination with morphine, buprenorphine, butorphanol, and nalbuphine produce additive or supra-additive effects on thermal nociception.

Experiment 2 examines the interactive effects of morphine in combination with metabotropic glutamate (mGlu) receptor antagonists selective for mGlu1, mGlu5, and mGlu2/3 receptor subtypes on schedule-controlled responding and thermal nociception. The results from this experiment suggest that mGlu1, mGlu5, and mGlu2/3 antagonists produce additive effects when administered in combination with morphine on schedule-controlled

responding. In contrast, mGlu1 and mGlu2/3 antagonists produced supra-additive effects with morphine when assessed on thermal nociception.

Experiment 3 assesses the effects of NMDA, mGlu1, mGlu5, and mGlu2/3 receptor antagonists on the efficacy of buprenorphine and dezocine in an assay of thermal nociception. Under conditions in which buprenorphine and dezocine produce sub-maximal antinociceptive effects, these drugs are assessed after pretreatment with NMDA, mGlu1, mGlu5, and mGlu2/3 antagonists. The results from this study indicate that NMDA, mGlu1, and mGlu2/3 receptor antagonists increase the efficacy of both buprenorphine and dezocine on thermal nociception.

Taken together, these experiments assessed the interactive effects of opioid receptor agonists and glutamate receptor antagonists on schedule-controlled responding and thermal nociception. The experimental results obtained from these studies suggest that these interactive effects are dependent upon factors such as the relative proportions of drugs and the experimental endpoint under study. In addition, the pharmacological affinity of the opioid and glutamate receptor antagonist being examined is an important determinant of their behavioral effects.

ACKNOWLEDGEMENTS

I would like to thank the members of my dissertation committee: Linda A. Dykstra, Clyde W. Hodge, Mark Hollins, Donald T. Lysle, and Mitchell J. Picker, for their valuable suggestions on this research project.

I am especially grateful to Linda Dykstra and Mitch Picker who provided endless support and guidance throughout these studies.

I am also indebted to my family: Mom, Dad, Chris, Kate, Nancy, Bill, and Brian. Thank you for the support and encouragement you have provided throughout this period.

Finally, I would like to thank my wife, Kelly. You have endured this project as much as I have and supported me throughout. Without your patience and sacrifice, none of this would be possible.

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LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
ANOVA	analysis of variance
cm	centimeter
C	Celsius
CL	confidence limits
ED_{50}	dose required to produce 50% of the maximal possible effect
g	gram
h	hour
iGlu	ionotropic glutamate
i.p.	intraperitoneal
kg	kilogram
MPE	maximum possible effect
mGlu	metabotropic glutamate
kainite	2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine
min	minute
mg	milligram
ml	milliliter
μl	microliter
NMDA	*N*-methyl-D-aspartate
PLC	phospholipase C
PLSD	protected least significant difference
s	second
S.E.M.	standard error of the mean
s.c.	subcutaneous

Z_{add}	predicted additive ED_{50} value
Z_{mix}	experimentally determined ED_{50} value

CHAPTER 1

GENERAL INTRODUCTION

GLUTAMATERGIC MODULATION OF THE BEHAVIORAL EFFECTS OF OPIOIDS

Glutamate is the most prominent excitatory neurotransmitter in the central nervous system. It is known to work through activation of both ionotropic (iGlu) and G-protein-coupled metabotrobic (mGlu) receptor subtypes. The three iGlu receptors were named after the agonists that activate them selectively: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainite). To date, eight mGlu receptor subtypes have been identified, and have been divided into three groups: group I (mGlu1 and mGlu5), group II (mGlu2 and mGlu3), and group III (mGlu4, mGlu6, mGlu7, and mGlu8).

The development of drugs with selectivity for iGlu and mGlu receptor subtypes has allowed for experiments assessing the role of glutamate in the behavioral effects of opioids. As a result of these studies, it is becoming increasingly clear that endogenous receptor activity at glutamate sites has a modulatory role in these effects. In support of this hypothesis, pharmacological antagonists with selectivity for iGlu and mGlu receptor subtypes modulate the effects of opioids on numerous behavioral endpoints. Among the iGlu and mGlu receptor subtypes, there is substantial evidence in support of a modulatory role of

the NMDA receptor and Group I and II mGlu receptors in the behavioral effects of the prototypical μ-opioid receptor agonist morphine.

To date, the most thoroughly investigated system of the glutamate receptor subtypes is the NMDA receptor. In what is now a seminal study, Trujillo and Akil (1991) first demonstrated that an NMDA receptor antagonist inhibits the development of morphine tolerance, suggesting that NMDA receptors have a central role in this effect. Subsequently, the interactive effects of NMDA receptor antagonists and morphine have been assessed on other endpoints. For example, several lines of evidence suggest that pharmacological antagonism of the NMDA receptor also blocks morphine-induced conditioned place preference (Papp et al., 2002), sensitization (Jeziorski et al., 1994), and physical dependence (Tanganelli et al., 1991), further suggesting that the effects of morphine can be modulated by NMDA receptors across a range of behaviors.

More recently, attention has turned to the interactive effects of opioids and mGlu receptor antagonists. Experimental evidence suggests that mGlu receptor antagonists modulate morphine's effects in a similar manner as ionotropic NMDA receptor antagonists on numerous behavioral endpoints. For example, co-administration of an mGlu receptor antagonist blocks morphine-induced conditioned place preference (Popik and Wrobel, 2002; Aoki et al., 2004), attenuates the development of morphine tolerance (Kozela et al., 2003; Smith et al., 2004) and attenuates the development of morphine dependence (Rasmussen et al., 2005) after repeated morphine administration. The results from these experiments are particularly exciting as they implicate alternative glutamate sites as modulators of morphine's behavioral effects.

Over the past ten years, our laboratory has focused on elucidating glutamatergic mechanisms in the antinociceptive effects of opioids. Throughout these studies, it has become increasingly clear that antagonists at the NMDA receptor enhance the acute antinociceptive effects of morphine and other opioids in a primate shock titration procedure (Allen and Dykstra, 2001; Allen et al., 2002; Allen et al., 2003), and in a mouse model of thermal nociception (Fischer et al., 2005). Together, these studies provided some of the first behavioral evidence to suggest that, in addition to other behavioral endpoints, NMDA receptor antagonists also increase the acute antinociceptive effects of opioids. To date, it remains to be determined if antagonists with selectivity for mGlu receptors also increase antinociception induced by opioid agonists.

One disadvantage of assessing glutamate antagonist/opioid combinations exclusively on an antinociceptive endpoint is that the results may lead to "false-positive" conclusions. For example, in preclinical assays of thermal nociception, a noxious stimulus is often presented that induces a specific withdrawal response (e.g., tail-flick from radiant heat, jump from a hot-plate surface). In these assays, a decrease in response latency after the presentation of the noxious stimulus is indicative of nociception and a subsequent increase in latency after administration of an opioid agonist is indicative of an antinociceptive response. Further, an increase in response latency that is quantitatively larger after administration of a glutamate receptor antagonist relative to the opioid alone is interpreted as an increase in opioid-induced antinociception. Clearly, alterations in response latency may be a result of alterations in other behaviors (motor function, sedation, etc.) that are unrelated to nociception. This observation is particularly important to address when examining glutamate receptor antagonists that can

produce marked effects on motor function when administered alone (Koek and Colpaert, 1990; Carter, 1994; Geter-Douglass and Witkin, 1999).

One particular advantage of the shock titration procedure used in some of the aforementioned studies is that the antinociceptive effects of NMDA antagonist/opioid agonist drug combinations are assessed in conjunction with their effects on operant rates of responding. Using this procedure, our laboratory has previously demonstrated that although NMDA antagonists increase the antinociceptive effects of morphine and other opioids, they do so without affecting rates of responding (Allen and Dykstra, 2001; Allen et al., 2002; Allen et al., 2003). These data suggest a separation between alterations in nociceptive behaviors and behaviors that are not related to nociception and further suggest that opioid-induced antinociception is not due to nonspecific motor effects. In addition, these previous studies demonstrate the importance of assessing the interactive effects of drugs across behavioral endpoints.

Together with reports from other laboratories assessing the interactive effects of opioids and NMDA antagonists (Mao et al., 1996; Bespalov et al., 1998; Pleasn et al., 1998; Bulka et al., 2002; Nemmani et al., 2004), the understanding of glutamatergic mechanisms in the acute antinociceptive effects of opioids has been enhanced. Taken together, these reports suggest that NMDA antagonists increase the acute antinociceptive effects of morphine and do so without affecting their rate-reducing effects (at least in the primate shock titration procedure). Less is know of the interactive effects of opioids and mGlu receptor antagonists on these endpoints. In addition, a formal quantitative analysis on these drug interactions has not been performed.

QUANTITATIVE METHODS FOR ASSESSING DRUG INTERACTIONS

If two drugs are administered concurrently, they may interact in an additive, infra-additive, or supra-additive manner. If two drugs produce behavioral effects through distinct cellular processes, the effects produced by their combination can be predicted to follow a dose-additive model. Briefly, this model predicts that the potency required of a mixture of the two drugs to produce a given effect is directly related to the potency of each drug administered alone.

In contrast, drugs that produce effects in an infra-additive or supra-additive manner produce effects that are less than or greater than expected, respectively, based on the individual potencies of the drugs administered alone. The detection of infra-additivity or supra-additivity suggests that some interaction has occurred between the pharmacokinetic or pharmacodynamic processes of the drugs. For example, one drug may affect the absorption, distribution, metabolism, or excretion of the other drug, resulting in an enhancement or a decrement its bioavailability. In addition, if the drugs are chemically related or act upon co-localized receptor sites, they may interact by altering drug affinity or efficacy, membrane function, or synaptic efficiency. Detection of this interaction may have both clinical and/or mechanistic implications.

Preclinical assessment of the effects of two drugs administered concurrently was first approached *graphically* by Loewe (1953). Loewe described a simple, yet eloquent graph, which represents the locus of equieffective dose pairs of two drugs in a mixture, or isobols, in accord with the potency of each drug administered alone (Figure 1.1). If two drugs are fully efficacious on the same endpoint, the dose of each required to produce a desired effect can be

denoted by A for drug A and B for drug B. Loewe's isobologram has Cartesian coordinates that represent equieffective combinations of the two drugs (a, b) that satisfy the following equation:

$$\frac{a}{A} + \frac{b}{B} = 1$$

For example, if a desired effect is achieved at a dose of 10 mg/kg of drug A (A = 10) and 5 mg/kg of drug B (B = 5), this leads to isobols of (8, 1), (6, 2), (4, 3), (2, 4), etc. The isobologram is also useful in displaying experimentally determined effects from mixtures of the two drugs, which can be compared to isobols that are predictive of dose-additivity. If experimentally determined doses required to produce a desired effect deviate from the line of additivity, it is suggestive of some interaction between the drugs. For example, if the desired effect is achieved in the above example at a dose of 3 mg/kg of drug A and 1 mg/kg of drug B, the coordinate (3, 1) would fall below the line of additivity. This is suggestive of a supra-additive interaction, as the desired effect was reached with lesser quantities of the drugs. In contrast, if the same effect required 10 mg/kg of drug A and 4 mg/kg of drug B, the coordinate (10, 4) would fall above the line of additivity, suggestive of an infra-additive interaction of the drugs. The isobologram is convenient for displaying the predicted and experimentally determined effects of drug mixtures. However, it does not contribute to the necessary statistical analysis that distinguishes deviation from additivity.

Advancement in *quantitative* methods for assessing the interactions of two drugs began with an analysis of the toxicity of poisons applied jointly. In what are now seminal papers, Bliss (1939) and Finney (1942) considered the problem of predicting the toxicity of a mixture of two drugs administered concurrently from the known dose-effect curves of each drug administered alone. These papers derived the necessary statistical analysis for the assessment

of drug mixtures, based on probit analysis, as well as the theoretical bases of the mixture's possible effects. However, the analysis described was not only complex, but was based on quantal data, consisting of the proportion of subjects responding to a particular dose. Thus, its application to behavioral endpoints, which are often based on continuous (graded) data sets, was limited.

Numerous methods have been employed to circumvent the statistical evaluation of data sets on an isobologram. One approach was utilized in a well publicized study by Gessner and Cabana (1970) on the interactive effects of chloral hydrate and ethanol. These authors assessed the effects of chloral hydrate, ethanol, and various mixtures of the two on the endpoints of loss of righting reflex and lethality. In this study, it was demonstrated that certain chloral hydrate/ethanol mixtures produced the desired effect at lower doses than expected, suggestive of a supra-additive interaction. Importantly, their findings suggest that the detection of synergism of two drugs depends on the relative proportions of the drugs, and the endpoint under study.

The statistical analysis utilized in the Gessner and Cabana (1970) study relied on plotting the coordinates of the experimentally determined doses together with their 95% confidence limits. Therefore, drug mixtures that produced the desired effect at doses with confidence limits that did not cross the theoretical dose-additive line were considered to deviate from additivity. This approach has since been used in the field of behavioral pharmacology (e.g., Woolverton and Balster, 1981; Foltin et al., 1983) and although it provided a quantitative dimension to the data sets, it was not supported by existing statistical methodology. Specifically, these studies did not recognize that the dose-additive line is an estimate based

on the experimentally determined dose-effect curve of each drug alone, and therefore is not free of error.

Drug combination studies in behavioral pharmacology have been discussed in reviews by Wessinger (1986) and Woolverton (1987), however it was noted that "no careful statistical evaluation [of dose-addition] has been published, and this is an area which needs further research" (Wessinger, 1986). Woolverton (1987) adds that "it remains for future research to refine the quantitative treatment of differences from additivity". More recently, the statistical analysis of the interactive effects of two drugs has been advanced by the work of Tallarida (1992, 2000).

A dose-addition analysis based on a fixed proportion design (Tallarida, 2000) can be applied to behavioral pharmacology questions. In this method, drug combinations are administered in amounts that keep the proportions of the drugs in the mixture constant, simplifying the analysis of the data. Escalating doses of the mixture of the two drugs can be tested at fixed proportions until the desired effect is reached. For example, consider the example previously described in which drug A produced a desired effect at 10 mg/kg and drug B produced the same effect at 5 mg/kg. If a proportion of 2:1 is selected, dose pairs (in mg/kg) of (2, 1), (4, 2), (6, 3) are administered until the desired effect is reached. The analysis is simplified so that the total dose in the mixture required to produce the effect is compared to the predicted effect in relation to the fraction (f) of its constitutes. The predicted additive dose (Z_{add}) can then be represented by the following equation:

$$Z_{add} = fA + (1 - f)B$$

From the experimentally determined total dose (Z_{mix}) and the predicted additive dose (Z_{add}), a statistical test of significance can be made (Z_{add} - Z_{mix}). If the difference is

significantly different from zero, it can be concluded that the drugs are interacting in a infra-additive ($Z_{add} < Z_{mix}$) or supra-additive ($Z_{add} > Z_{mix}$) manner. This approach is applicable to graded data sets, and has recently been utilized to quantify the interactive effects of two drugs on numerous behavioral endpoints (e.g., Stevenson et al., 2003, 2005; Negus, 2005; Craft and Leitl, 2006). From the development of rigorous quantitative analyses for the assessment of drug interactions, a comprehensive behavioral assessment of NMDA antagonist/opioid and mGlu antagonist/opioid drug mixtures will advance our understanding of drug mechanism as well as shed light on the possible clinical utility of these drug combinations.

IONOTROPIC AND METABOTROPIC GLUTAMATE RECEPTOR PHARMACOLOGY

The conclusions gleaned from studies assessing of the role of glutamate receptor subtypes in the antinociceptive effects of opioids are only as valid as the selectivity of the glutamate receptor antagonists employed in these studies. Clearly, it is important to take advantage of the most recent advances in glutamate receptor pharmacology. Since the discovery of iGlu receptors in the late 1950's and mGlu receptors by Sladeczek and colleagues in 1985, there has been considerable progress in the development of drugs that are selective for iGlu and mGlu receptor subtypes. The identification of iGlu and mGlu receptor antagonists in particular has enabled considerable advances in the understanding of the role of glutamate in the behavioral effects of morphine.

Pharmacological antagonists selective for iGlu and mGlu receptor subtypes can be divided into competitive and noncompetitive antagonists. Competitive antagonists selective for the NMDA receptor have high affinity for either the glutamate or glycine recognition site,

whereas noncompetitive antagonists block the ion channel pore of the receptor. Although noncompetitive antagonists have been approved for human use (Farlow, 2004), these drugs often have steep dose-response curves and low therapeutic indexes (Kew and Kemp, 1998), limiting their clinical utility. Of the numerous competitive antagonists available, (-)-6-phosphonomethyl-deca-hydroisoquinoline-3-carboxylic acid (LY235959) is a potent and highly selective NMDA antagonist with affinity for the glutamate site on the NMDA receptor complex (Schoepp et al., 1991).

Although the pharmacokinetic profile of competitive Group I mGlu receptor antagonists has restricted their utility in vivo (Kew and Kemp, 2005), selective noncompetitive antagonists (allosteric modulators) have been developed. To date, only (3aS,6aS)-6a-naphtalan-2-ylmethyl-5-methyliden-hexahydro-cyclopenta[c]furan-1-on (BAY 36-7620) and more recently (3,4-Dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone (JNJ16259685) have been shown to be centrally active at mGlu1 receptors, with JNJ16259685 showing increased potency over BAY 36-7620 (Carroll et al., 2001; Lavreysen et al., 2004). The first noncompetitive mGlu5 receptor antagonists to be described were 6-methyl-2-(phenylazo)-3-pyridinol (SIB-1757) and (*E*)-2-methyl-6-(2-phenylethenyl)-pyridine (SIB-1893), which are selective over other mGlu receptor subtypes (Varney et al., 1999). Subsequently, a more potent and selective mGlu5 receptor antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) has been identified (Gasparini et al., 1999).

Group II mGlu receptor antagonists selective for either mGlu2 or mGlu3 receptors have not been synthesized. The most potent mGlu2/3 receptor antagonist identified to date is 2*S*-2-amino-2-(1*S*,2*S*-2-carboxycycloprop-1-yl)-3-(xanth-9-yl) propanoic acid (LY341495). As a competitive antagonist at mGlu2/3 receptors, LY341495 interacts with the extracellular N-

terminal binding domain, inhibiting receptor function (Kingston et al., 1998; Kew and Kemp, 2005).

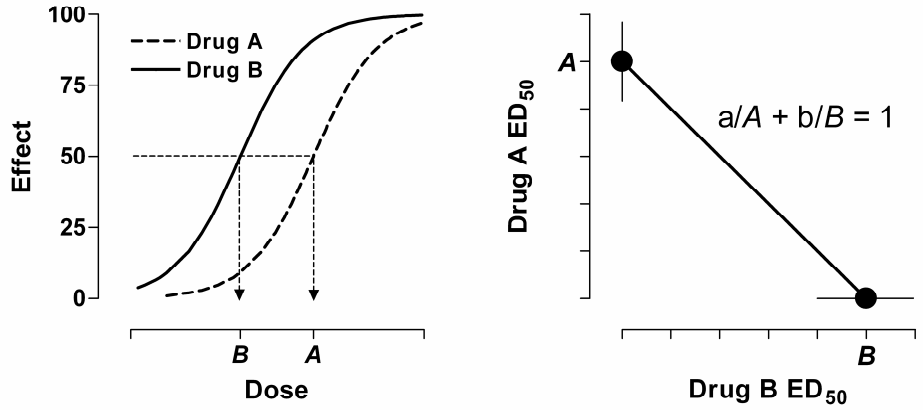
EXPERIMENTAL AIMS

The purpose of the present series of experiments is to examine the interactive effects of opioids and glutamate receptor antagonists in assays of schedule-controlled responding and thermal nociception. Experiment 1 examines the effects of morphine, buprenorphine, butorphanol, and nalbuphine alone and in combination with the NMDA receptor antagonist LY235959. Although previous reports have assessed the interactive effects of opioids and NMDA receptor antagonists on thermal nociception, the first purpose of this study is to analyze these interactions using a formally quantitative approach. Dose addition analysis is used to differentiate effects that are additive from effects that are infra-additive or supra-additive. The second purpose of this study is to determine whether these interactions extend to another behavioral endpoint. An assay of schedule-controlled responding is used to assess the extent to which LY235959 selectively increases opioid-induced antinociception.

Experiment 2 examines the interactive effects of morphine in combination with metabotropic glutamate (mGlu) receptor antagonists selective for mGlu1 (JNJ16259685), mGlu5 (MPEP), and mGlu2/3 (LY341495) receptor subtypes in assays of schedule-controlled responding and thermal nociception. These studies provide the first systematic analysis of the interactive effects of mGlu receptor antagonists and morphine on these endpoints. Drug interactions are analyzed with the quantitative methods used in Experiment 1 to differentiate effects that are additive from effects that are infra-additive or supra-additive.

The purpose of Experiment 3 is to use opioid efficacy as a tool to dissociate the mechanism by which mGlu receptor antagonists increase the antinociceptive effects of morphine and other opioids. In this experiment, morphine and the low-efficacy μ-opioid receptor agonists buprenorphine and dezocine are first assessed in an assay of thermal nociception under conditions of low (53°C) and high (56°C) stimulus intensity. Under conditions in which the low-efficacy μ-opioid receptor agonists produce sub-maximal effects, these drugs are assessed after pretreatment with LY235959, JNJ16259685, MPEP, and LY341495.

Figure 1.1 Hypothetical dose-effect curves and isobologram. Left, dose-effect curves for fully efficacious drugs A and B. Arrows highlight the ED_{50} value for each drug. Right, isobologram for mixtures of drug A and drug B at the 50% effect level. Abscissae, ED_{50} value for drug B. Ordinate, ED_{50} value for drug A.



CHAPTER 2

EXPERIMENT 1: INTERACTIONS BETWEEN AN NMDA ANTAGONIST AND LOW-EFFICACY OPIOID RECEPTOR AGONISTS IN ASSAYS OF SCHEDULE-CONTROLLED RESPONDING AND THERMAL NOCICEPTION

INTRODUCTION

A substantial literature has implicated *N*-Methyl-D-Aspartate (NMDA) receptor mechanisms in the acute antinociceptive effects of morphine. For example, behavioral evidence suggests that pretreatment or coadministration of an NMDA receptor antagonist can increase the acute effects of morphine in animal models of thermal nociception (Allen and Dykstra, 2003; Nemmani et al., 2004; Fischer et al., 2005). In addition, NMDA antagonists can increase morphine-induced analgesia in humans under certain conditions (Javery et al., 1996; Bossard et al., 2002; Suzuki et al., 2005).

Although NMDA receptor involvement in morphine-induced antinociception has received considerable attention, less is known of NMDA receptor involvement in antinociception induced by low-efficacy opioids. To date, a single study has assessed these interactions, showing that coadministration with the competitive NMDA antagonist LY235959 can increase the antinociceptive activity of the low-efficacy opioids buprenorphine and butorphanol in monkeys responding under a schedule of shock titration, suggesting NMDA receptor involvement in these effects (Allen et al., 2003). This latter

finding is particularly intriguing as the enhancement of the antinociceptive effects of low-efficacy opioids may increase their clinical utility.

Taken together, these results suggest that inhibition of the NMDA receptor may increase the antinociceptive effects of morphine and certain low-efficacy agonists. Although these data implicate the NMDA receptor system in opioid antinociception, these interactions have not been quantified precisely in order to determine deviation from simple additivity. The use of dose-addition analysis can provide a more quantitative evaluation of drug interactions and can be used to differentiate effects that are additive from effects that are infra-additive or supra-additive (Wessinger, 1986; Tallarida, 2000).

In the present study, dose-addition analysis was used to further evaluate NMDA/opioid interactions. The effects of combinations of the NMDA receptor antagonist LY235959 and various opioid receptor agonists were examined in C57BL/6 mice using two different assays. To assess the extent to which NMDA antagonists selectively increase opioid-induced antinociception, the rate-decreasing effects of LY235959/opioid mixtures were first examined in an assay of schedule-controlled responding maintained by liquid food. Second, the antinociceptive effects of LY235959/opioid mixtures were examined in an assay of thermal nociception. Drug interactions were assessed using a fixed-proportion design, as this has been recommended for the study of drug interactions (Wessinger, 1996; Tallarida, 2000) and has been used to study similar drug mixtures on similar endpoints (Stevenson et al., 2003, 2005).

The opioid receptor agonists examined were morphine and the low-efficacy agonists buprenorphine, butorphanol, and nalbuphine as these are each structurally diverse drugs that differ in pharmacological selectivity, specifically their affinity for the μ-opioid receptor and

κ-opioid receptor (e.g. Chen et al., 1992; Walker et al., 1994, 1998; Emmerson et al., 1996). LY235959, the active isomer of LY274614, was selected because it is a potent NMDA antagonist with high selectivity for the competitive site on the NMDA receptor complex (Schoepp et al., 1991).

METHODS

Animals. Adult male C57BL/6 mice weighing between 26 and 34 g were purchased from Jackson Labs (Raleigh, N-C). Upon arrival, mice were group housed in standard plexiglas cages in a colony room maintained on a 12-h light/dark cycle (lights on at 7:00 PM). All mice had continuous access to food and water throughout the study and were habituated to the colony room environment for 2 weeks prior to any experimental manipulation. Mice were also exposed to the testing environment and handled for two days prior to initiation of an experiment. All testing procedures were conducted between 11:00 AM and 3:00 PM. Throughout all testing the "Guide for the Care and Use of Laboratory Animals" (National Research Council, National Academy of Sciences, Washington, D.C., 1996) was adhered to.

Drugs. Morphine sulfate and buprenorphine hydrochloride were provided by NIDA (Bethesda, MD) and LY235959 by Lilly Research Laboratories (Indianapolis, IN). Butorphanol tartate and nalbuphine hydrochloride were purchased from Sigma (St.Louis, MO). All drugs were dissolved in 0.9% phosphate buffered saline. Drugs were injected i.p. at a volume of 0.1 ml/10 g.

Schedule-Controlled Responding. Response rates in the assay of schedule-controlled responding were assessed in an experimental operant chamber (approximately 14 cm×14 cm×14 cm) equipped with a house light, ventilator fan, and two nosepoke holes (1.2 cm diameter) that were located on either side of a liquid dipper. The operant chamber was controlled by a MED-PC interface and an IBM compatible computer programmed with MED Associates software (MED Associates, St. Albans, VT).

Mice were trained under a multiple-cycle procedure during experimental sessions conducted 5 days each week. Each training cycle consisted of a 10-min pretreatment period

followed by a 5-min response period. During the pretreatment period, stimulus lights were not illuminated and responding had no scheduled consequences. During the response period, the right nosepoke was illuminated and mice could obtain up to 10 liquid food reinforcers (8 s access to 8 μl Ensure®) under a fixed ratio 3 schedule of food presentation. If all 10 reinforcers were earned before 5 min had elapsed, the light was turned off, and responding had no scheduled consequences for the remainder of the response period. The left nosepoke was inactive, and responding at this hole had no scheduled consequences. Training sessions consisted of five consecutive cycles, and testing began once response rates were stable throughout the session.

Test sessions replaced the last training session of each week if responding was stable throughout the preceding training sessions. Test sessions were identical to training sessions except that cumulative doses of drug mixtures were administered i.p. during the first minute of the pretreatment period of each cycle (i.e., 15-min inter injection interval), increasing in one-quarter or one-half log unit increments. Data are expressed as a percentage of control responding using the average rate of responding from the previous day as the control value (average of 5 cycles).

Thermal Nociception. Antinociception was assessed with a tail-flick analgesia meter (Columbus Instruments, Columbus, OH). During this procedure, the stimulus intensity was adjusted to provide baseline latencies between 3-5 s. The antinociceptive response was evaluated by recording the latency to flick the tail from the light source. Responses were measured using a stopwatch to the nearest 0.1 s. A predetermined cutoff time of 10 s was defined as a maximal response and was employed to prevent tissue damage. Immediately following the termination of a trial, mice were removed from the apparatus and returned to

the homecage. The latency to respond to the light source was measured twice at each determination, at least 30 s apart, at 3 and 5 cm from the tip of the tail. These data were averaged to yield one value. Following baseline latency measurements, multiple 15 min cycles were run and drugs mixtures were administered cumulatively. During this procedure, cumulative doses of drug mixtures were administered i.p. during the first min of each cycle (i.e., 15-min inter injection interval), increasing in one-quarter or one-half log unit increments and antinociceptive measurements were determined during the last minute of each cycle. Latencies are expressed as a percentage of the maximal possible effect (%MPE) using the following formula: %MPE = [postdrug latency (seconds) - baseline latency (seconds)] / [cutoff time (10 s) - baseline latency (seconds)].

Data analysis. The dose of each drug mixture required to produce a 50% decrease in responding (ED_{50}) in the assay of schedule-controlled responding was derived using log-linear interpolation by linear regression. The dose of each drug mixture required to produce 50% maximum antinociceptive effect was derived in a similar manner. In each assay, ED_{50} values were determined using the linear portion of the dose-effect curve up to doses that produced a maximal effect.

Interactions between LY235959 and opioid agonists were assessed using both graphical and statistical approaches (Wessinger, 1986; Tallarida, 2000). Graphically, the distinction between infra-additive, additive, or supra-additive interactions were made with the use of isobolograms. In the current study, isobolograms were constructed by connecting the ED_{50} of LY235959 alone plotted on the abscissa with the ED_{50} of the opioid receptor agonist alone plotted on the ordinate to obtain an additivity line. The additivity line contains the loci of dose pairs that produce an ED_{50} equal to the ED_{50} of LY235959 or an opioid receptor agonist

alone. Dose pairs that fall below the additivity line suggest an ED_{50} was reached with lesser quantities of the drugs, suggestive of synergism. In contrast, experimental points representing dose pairs that fall above the line are suggestive of subadditivity.

Isobolograms of dose pairs (a,b) were also constructed based on the relative efficacy of LY235959 and an opioid receptor agonist. Each opioid was fully efficacious in both assays, and was used as the reference drug. Therefore, the shape of the isobologram is dependent on the efficacy of LY235959 from the equation

$$B = b + \frac{B}{\left(\frac{100}{A_{\max}}\right)\left(1 + \frac{A_c}{a}\right) - 1}$$

where B is the ED_{50} for an opioid alone, $A_{\max}$ is related to the efficacy of LY235959, and A_c is the dose of LY235959 that attains half of $A_{\max}$ (Grabovsky and Tallarida, 2004). For the assay of schedule-controlled responding, in which all drugs were fully efficacious, the equation describing the isobologram is reduced to

$$B = b + \frac{B}{\left(\frac{A}{a}\right)} \quad \text{or} \quad \frac{a}{A} + \frac{b}{B} = 1 .$$

For the assay of thermal nociception, LY235959 was ineffective when administered alone. Therefore, as the value of $A_{\max}$ approaches 0, the equation describing this isobologram reduces to

$$B = \lim_{A_{\max} \to 0} \left\{ b + \frac{B}{\left(\frac{100}{A_{\max}}\right)\left(1 + \frac{A_c}{a}\right) - 1} \right\} \quad \text{or} \quad B = b .$$

Drug interactions were statistically analyzed by comparing the experimentally determined ED_{50} values for each mixture (Z_{mix}) with predicted additive ED_{50} values (Z_{add}) as described by Tallarida (2000). Z_{mix} was defined as the total drug dose (i.e., dose LY235959 + dose opioid receptor agonist) that produced a 50% decrease in rates of responding (assay of schedule-controlled responding) or a 50% maximum antinociceptive effect (assay of thermal nociception).

For the assay of schedule-controlled responding, in which all drugs were equieffective, Z_{add} values were calculated individually for each mouse based on the ED_{50} values of each drug from the equation

$$Z_{add} = fA + (1 - f)B$$

where A is the ED_{50} for LY235959 alone and B is the ED_{50} for the opioid receptor agonist alone. The proportion of LY235959 (ρ_A) in each mixture is determined by the equation

$$\rho_A = \frac{fA}{fA + (1 - f)B}.$$

The present study examined effects produced by mixtures in which f = 0.25, 0.5, and 0.75. When f = 0.25, the mixture contains a proportion of [A/ (A + 3B)] LY235959 and a mixture ratio of [(A/ B) ÷ 3] parts LY235959 to 1 part opioid receptor agonist; f = 0.50 leads to a proportion of [A/ (A + B)] LY235959 in the mixture and a mixture ratio of (A/ B) parts LY235959 to 1 part opioid agonist; and f = 0.75 leads to a proportion of [A/ (A + B/ 3)] LY235959 in the mixture and a mixture ratio of [(A/ B) x 3] parts LY235959 to 1 part opioid receptor agonist.

For the assay of thermal nociception LY235959 alone was ineffective, and the hypothesis of additivity predicts that LY235959 would not contribute to the effects of a mixture of LY235959 in combination with an opioid receptor agonist (Tallarida, 2000). Therefore, Z_{add}

is calculated based on the proportion of opioid agonist (ρ_B) in each particular mixture from the equation

$$Z_{add} = \frac{B}{\rho_B}.$$

Mean experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) for each mixture were compared with a t-test.

Data were further analyzed by calculating opioid ED_{50} values for each opioid receptor agonist alone and in combination with various proportions of LY235959. A dose ratio was defined as the ED_{50} value of the opioid receptor agonist alone divided by the ED_{50} value of the opioid receptor agonist in the mixture. Dose ratios were determined for each LY235959/opioid agonist mixture in both the assay of schedule-controlled responding and the assay of thermal nociception. The dose ratios for each mixture were then compared between assays (Assay Ratio) according to the equation: [Dose Ratio (thermal nociception)] ÷ [Dose Ratio (schedule-controlled responding)].

RESULTS

Schedule-controlled responding.

LY235959 and opioid agonists alone. Figure 2.1 (left panel) shows the rate-decreasing effects of LY235959, morphine, buprenorphine, butorphanol, and nalbuphine. Each drug produced dose-dependent decreases in the rate of responding resulting in ED_{50} values (± 95% confidence limits) of 1.4 (0.97-1.9) for morphine, 0.11 (0.088-0.15) for buprenorphine, 0.15 (0.088-0.24) for butorphanol, and 4.2 (1.6-11) for nalbuphine. Therefore, the relative potencies for the opioid agonists in the assay of schedule-controlled responding was buprenorphine = butorphanol > morphine = nalbuphine. Dose-effect curves for LY235959 were determined separately for each group resulting in ED_{50} values (± 95% confidence limits) of 5.2 (3.7-7.4), 6.1 (3.4-11), 6.6 (3.5-12), and 5.8 (3.9-8.7), and the relative potencies of these values were used to determine relative proportions of the compounds used in subsequent studies for morphine, buprenorphine, butorphanol, and nalbuphine, respectively (see Data Analysis).

LY235959 and opioid agonist mixtures. The rate-decreasing effects of each opioid agonist alone and in combination with LY235959 are shown in Figure 2.2. Each drug mixture produced dose-dependent decreases in response rates. Addition of LY235959 produced leftward shifts in the dose-effect curves for morphine and buprenorphine, and the magnitude of shift was correlated with the proportion of LY235959 in the mixture. Figure 2.2 also shows the graphical analysis of these drug combinations. Mixtures with a lower proportion of LY235959 relative to morphine (i.e., 1.3:1 LY235959/morphine and 3.9:1 LY235959/morphine) or to buprenorphine (i.e., 18:1 LY235959/buprenorphine) produced infra-additive effects, as these ED_{50} values fell above the line of additivity. Mixtures with a

higher proportion of LY235959 relative to morphine and buprenorphine produced additive effects, as these ED_{50} values fell close to the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} > Z_{mix}$ or $Z_{add} = Z_{mix}$) (Table 2.1).

Addition of LY235959 also produced leftward shifts in the dose-effect curves for butorphanol and nalbuphine, and the magnitude of shift was correlated with the proportion of LY235959 in the mixture. Graphical analysis of these drug combinations indicates that mixtures with a lower proportion of LY235959 relative to butorphanol and nalbuphine produced additive effects, as these ED_{50} values fell close to the line of additivity. Mixtures with a higher proportion of LY235959 relative to butorphanol (i.e., 140:1 LY235959/butorphanol) or to nalbuphine (i.e., 1.4:1 LY235959/nalbuphine and 4.1:1 LY235959/ nalbuphine) produced supra-additive effects, as these ED_{50} values fell below the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (Table 2.1).

Thermal nociception.

LY235959 and opioid agonists alone. Figure 2.1 (right panel) shows the antinociceptive effects of LY235959, morphine, nalbuphine, buprenorphine, and butorphanol. LY235959 was without effect in this assay up to a dose of 10 mg/kg, which produced severe motor impairment. Each opioid receptor agonist produced dose-dependent increases in latency to respond to the tail-flick apparatus, resulting in ED_{50} values (± 95% confidence limits) of 3.5 (2.3-5.1) for morphine, 0.25 (0.21-0.31) for buprenorphine, 0.12 (0.078-0.19) for butorphanol, and 3.4 (2.1-5.5) for nalbuphine. The relative potencies for the opioid agonists in the assay of thermal nociception was similar to the relative potencies determined in the

assay of schedule-controlled responding (butorphanol = buprenorphine > nalbuphine = morphine). LY235959 was without effect in this assay, therefore the relative potencies determined in the assay of schedule-controlled responding were used to determine the relative proportions of the compounds in each mixture.

LY235959 and opioid agonist mixtures. The antinociceptive effects of the opioid receptor agonists alone and in combination with LY235959 are shown in Figure 2.3. Each drug mixture produced dose-dependent increases in antinociception. Addition of LY235959 produced leftward shifts in the dose-effect curves for each of the opioid receptor agonists, and the magnitude of shift was correlated with the proportion of LY235959 in the mixture. Graphical analysis of these drug combinations indicates that each mixture with a relatively lower proportion of LY235959 to morphine (i.e., 1.3:1 LY235959/morphine), buprenorphine (i.e., 18:1 LY235959/buprenorphine), or butorphanol (i.e., 15:1 LY235959/butorphanol) produced supra-additive effects, while the effects of the LY235959/nalbuphine mixture was additive. Mixtures with an intermediate or higher proportion of LY235959 relative to all four of the opioid receptor agonists examined also produced supra-additive effects, as these ED_{50} values fell to the left of the line of additivity. Statistical comparison determined that the experimentally determined ED_{50} values (Z_{mix}) for these mixtures were less than the predicted additive ED_{50} values (Z_{add}) (Table 2.2).

Tables 2.3, 2.4, and 2.5 show the dose ratios obtained in the assay of schedule-controlled responding, the dose ratios obtained in the assay of thermal nociception, and the ratios between these assays for each LY235959/opioid agonist mixture. In the assay of schedule-controlled responding, the addition of the lesser proportion LY23959 to morphine, buprenorphine and butorphanol produced rightward shifts in the opioid dose curves relative

to the opioid alone. Increasing proportions of LY235959 in the mixture of these drugs produced proportion-dependent leftward shifts in the opioid dose-effect curves. The addition of LY235959 to nalbuphine produced leftward shifts in the nalbuphine dose-effect curve at all proportions in a proportion-dependent manner. In the assay of thermal nociception, the addition of LY235959 to the mixture of each opioid agonist produced leftward shifts in the opioid dose-effect curves. For each drug tested, the dose-effect curves were shifted in a proportion-dependent manner with the exception of morphine and butorphanol, where the intermediate proportion of morphine was shifted the least relative to the LY235959/morpine mixtures and the intermediate proportion of butorphanol was shifted to the greatest extent relative to the LY235959/butorphanol mixtures.

Assay ratios were determined as a measure of the degree to which addition of LY235959 shifted the opioid dose-effect curve in the assay of thermal nociception relative to the assay of schedule-controlled responding. The addition of LY235959 to morphine, buprenorphine, and butorphanol produced greater shifts in the opioid dose-effect curves in the assay of thermal nociception relative to the assay of schedule-controlled responding across all proportions. Conversely, LY235959 shifted the nalbuphine dose-effect curve to a greater extent in the assay of schedule-controlled responding relative to the assay of thermal nociception.

DISCUSSION

The present study provides a quantitative assessment of the degree to which the NMDA antagonist LY235959 can modulate the antinociceptive and rate-decreasing effects of morphine, buprenorphine, butorphanol, and nalbuphine. Previous research has demonstrated that NMDA antagonists can increase the antinociceptive effects of morphine (Allen and Dykstra, 2003; Nemmani et al., 2004; Fischer et al., 2005; Grisel et al., 2005). Although the interactive effects of morphine and NMDA receptor antagonists have been investigated in various antinociceptive measures, the first purpose of the current study was to assess these interactions using a formally quantitative approach. The quantitative assessment of behavior using dose-addition analysis was used to distinguish interactive effects of morphine and LY235959 that were additive from effects that were infra-additive or supra-additive.

A second purpose was to determine if NMDA/opioid interactions extend to another behavioral endpoint. Specifically, the effects of NMDA/opioid mixtures unrelated to antinociception can be addressed systematically by, for example, comparing doses required to produce antinociception to doses that eliminate food maintained responding. Therefore, an assay of schedule-controlled responding was used to determine the selectivity of the drug combinations to produce antinociceptive effects vs. nonspecific effects.

A third purpose of the present study was to extend these findings to opioids with lower efficacy that vary in pharmacological selectivity. Behavioral evidence with opioid antagonists suggest the rate-decreasing and antinociceptive effects of the drugs selected for analysis in the current study are mediated through actions at opioid receptors (e.g. Zimmerman et al., 1987; Pitts et al., 1996). Furthermore, similar studies have demonstrated differences in the relative efficacies between these opioid receptor agonists (Zimmerman et

al., 1987; Adams et al., 1990; Walker et al., 1998). In vitro studies confirm these findings, suggesting that buprenorphine, butorphanol, and nalbuphine are lower in efficacy relative to morphine (Chen et al., 1992; Toll, 1995; Emmerson et al., 1996).

In the current study, three different proportions of LY235959 to each opioid agonist were studied as it has been suggested that deviation from additivity depends on the relative proportions of the drugs under study (Gessner and Cabana, 1970; Tallarida, 2000). In agreement with these findings, the nature of the LY235959/opioid interactions in the assay of schedule-controlled responding was dependent on the proportion of LY235959 in each mixture. For example, the LY235959/morphine mixture with the higher proportion of LY235959 produced additive effects in this assay, whereas LY235959/morphine mixtures with a relatively lower proportion of LY235959 produced infra-additive effects. Similar to LY235959/morphine mixtures, the effects of LY235959/buprenorphine mixtures were either additive or infra-additive, depending on the proportions of each of the drugs.

In addition to the relative proportions of drugs in a mixture, the effect of an interaction of two drugs may depend on the experimental endpoint under study (Stevenson et al., 2003, 2005). In agreement with these findings, LY235959 produced a supra-additive interaction with each of the opioids, regardless of efficacy, in the assay of thermal nociception. These findings agree with data obtained in squirrel monkeys (Allen et al., 2003), suggesting efficacy is not an important determinant of interactions between NMDA and opioid receptors on the endpoint of antinociception.

If the mechanism of action of each of the drugs in a mixture is mediated through different receptors, the detection of synergism suggests an interaction between their receptor mediated signals (Tallarida, 2000). In the current study, LY235959 and each of the low-efficacy

opioids produced supra-additive effects in the assay of thermal nociception. Previous research has suggested a model of NMDA receptor activation contributing to neural and behavioral plasticity that may underlie the alterations in the antinociceptive effectiveness of morphine. According to this model, administration of morphine activates NMDA receptors through intracellular mechanisms, increasing intracellular calcium levels, leading to an increase in protein kinase C and subsequent reduction in the sensitivity of μ-opioid receptors (Mao et al., 1995). The current study suggests that similar receptor mediated interactions may occur with opioids of lower efficacy.

The present study demonstrates that studying drug interactions across a range of relative proportions and across experimental endpoints can reveal characteristics of these interactions that may have clinical potential. For example, in the assay of schedule-controlled responding, the LY235959/buprenorphine mixture with the lowest proportion of LY235959 produced sub-additive effects in the assay of schedule-controlled responding while producing supra-additive effects in the assay of thermal nociception. This LY235959/buprenorphine mixture resulted in a favorable ED_{50} ratio across experimental endpoints confirming a greater increase in buprenorphine's antinociceptive effects relative to its rate-decreasing effects. This finding was similar to LY235959/morphine mixtures, and suggests that LY235959 may specifically increase the antinociceptive properties of these drugs relative to their nonspecific effects.

Drugs such as buprenorphine, butorphanol and nalbuphine have a lower potential for abuse and exert less side effects relative to morphine (Hoskin and Hanks, 1991; Preston and Jasinski, 1991), however they also are less effective, at least in animal models of antinociception (Dykstra 1990; Walker, et al. 1993; Morgan, et al. 1999). If NMDA

antagonists potentiate the antinociceptive effects of low-efficacy opioids without increasing opioid-induced side effects, combination treatment might be useful for the management of various pain states. Therefore, manipulation of pharmacological selectivity and relative concentrations of low-efficacy opioids in a drug cocktail may enhance clinical utility. Further characterization of NMDA antagonist/opioid agonist interactions are necessary to determine their interactive effects on other behavioral endpoints (such as respiratory depression and self-administration) and to determine if the effects of low-efficacy opioids are modulated by an NMDA antagonist other than LY235959.

Table 2.1 Predicted additive ED_{50} values (Z_{add}) and experimentally determined ED_{50} values (Z_{mix}) of mixtures of LY235959 administered in combination with opioid agonists in the assay of schedule-controlled responding.

*, Z_{mix} significantly different from Z_{add} ($p < .05$).

Drug Mixture	Z_{add} (± 95% CL)	Z_{mix} (± 95% CL)
LY235959 + Morphine		
1.3:1 LY235959/Morphine	2.2 (1.7-3.0)	4.5 (3.3-6.5)*
3.9:1 LY235959/Morphine	3.1 (2.4-4.0)	5.1 (4.2-6.2)*
12:1 LY235959/Morphine	3.9 (2.9-5.1)	3.7 (2.7-5.1)
LY235959 + Buprenorphine		
18:1 LY235959/Buprenorphine	1.5 (0.98-2.3)	2.8 (2.2-3.4)*
54:1 LY235959/Buprenorphine	2.9 (1.9-4.4)	3.1 (2.2-4.3)
160:1 LY235959/Buprenorphine	4.2 (2.7-6.5)	5.5 (4.1-7.2)
LY235959 + Butorphanol		
15:1 LY235959/Butorphanol	1.9 (1.1-3.3)	2.8 (1.7-4.5)
45:1 LY235959/Butorphanol	3.5 (2.0-6.1)	2.8 (1.4-5.8)
140:1 LY235959/Butorphanol	5.1 (2.9-9.0)	1.9 (1.0-3.6)*
LY235959 + Nalbuphine		
0.46:1 LY235959/Nalbuphine	4.9 (2.2-11)	1.8 (0.91-3.6)
1.4:1 LY235959/Nalbuphine	5.7 (3.3-9.8)	1.4 (0.80-2.7)*
4.1:1 LY235959/Nalbuphine	5.9 (4.3-8.3)	0.67 (0.099-4.5)*

Table 2.2 Predicted additive ED_{50} values (Z_{add}) and experimentally determined ED_{50} values (Z_{mix}) of mixtures of LY235959 administered in combination with opioid agonists in the assay of thermal nociception.

*, Z_{mix} significantly different from Z_{add} ($p < .05$).

Drug Mixture	Z_{add} (± 95% CL)	Z_{mix} (± 95% CL)
LY235959 + Morphine		
1.3:1 LY235959/Morphine	7.7 (5.0-12)	1.4 (0.43-4.5)*
3.9:1 LY235959/Morphine	16 (11-25)	5.5 (3.4-8.8)*
12:1 LY235959/Morphine	42 (28-64)	5.7 (2.6-13)*
LY235959 + Buprenorphine		
18:1 LY235959/Buprenorphine	4.7 (4.0-5.6)	1.5 (1.2-1.9)*
54:1 LY235959/Buprenorphine	14 (11-16)	2.9 (1.2-7.0)*
160:1 LY235959/Buprenorphine	40 (34-48)	5.5 (3.4-8.9)*
LY235959 + Butorphanol		
15:1 LY235959/Butorphanol	1.9 (0.96-3.8)	0.34 (0.12-1.0)*
45:1 LY235959/Butorphanol	5.5 (2.8-11)	0.63 (0.24-1.7)*
140:1 LY235959/Butorphanol	16 (8.2-32)	1.3 (0.46-3.5)*
LY235959 + Nalbuphine		
0.46:1 LY235959/Nalbuphine	4.8 (2.7-8.4)	4.3 (2.6-6.9)
1.4:1 LY235959/Nalbuphine	7.8 (4.4-14)	2.8 (1.1-7.1)*
4.1:1 LY235959/Nalbuphine	17 (9.5-29)	4.6 (2.8-7.4)*

Table 2.3 ED_{50} values and dose ratios of opioids alone and in mixtures with LY235959 in the assay of schedule-controlled responding.

*, Significantly different from opioid alone

Drug Mixture	ED_{50} (± 95% CL)	Dose Ratio
Morphine Alone	1.4 (0.97-1.9)	
1.3:1 LY235959/Morphine	2.4 (1.8-3.4)	0.58
3.9:1 LY235959/Morphine	1.1 (0.73-1.7)	1.3
12:1 LY235959/Morphine	0.31 (0.24-0.42)*	4.5
Buprenorphine Alone	0.11 (0.088-0.15)	
18:1 LY235959/Buprenorphine	0.15 (0.11-0.19)	0.73
54:1 LY235959/Buprenorphine	0.061 (0.032-0.12)	1.8
160:1 LY235959/Buprenorphine	0.035 (0.024-0.52)	3.1
Butorphanol Alone	0.15 (0.088-0.24)	
15:1 LY235959/Butorphanol	0.16 (0.096-0.28)	0.94
45:1 LY235959/Butorphanol	0.080 (0.066-0.10)	1.9
140:1 LY235959/Butorphanol	0.018 (0.013-0.025)*	8.3
Nalbuphine Alone	4.2 (1.6-11)	
0.46:1 LY235959/Nalbuphine	2.2 (0.75-6.2)	1.9
0.46:1 LY235959/Nalbuphine	0.65 (0.38-1.1)*	6.5
1.4:1 LY235959/Nalbuphine	0.14 (0.035-0.59)*	30

Table 2.4 ED_{50} values and dose ratios of opioids alone and in mixtures with LY235959 in the assay of thermal nociception.

*, Significantly different from opioid alone

Drug Mixture	ED_{50} (± 95% CL)	Dose Ratio
Morphine Alone	3.5 (2.3-5.1)	
1.3:1 LY235959/Morphine	0.87 (0.51-1.5)*	4.0
3.9:1 LY235959/Morphine	1.2 (0.82-1.7)*	2.9
12:1 LY235959/Morphine	0.48 (0.20-1.2)*	7.3
Buprenorphine Alone	0.25 (0.21-0.31)	
18:1 LY235959/Buprenorphine	0.082 (0.067-0.10)*	3.0
54:1 LY235959/Buprenorphine	0.070 (0.053-0.091)*	3.6
160:1 LY235959/Buprenorphine	0.038 (0.021-0.070)*	6.6
Butorphanol Alone	0.12 (0.078-0.19)	
15:1 LY235959/Butorphanol	0.034 (0.022-0.053)*	3.5
45:1 LY235959/Butorphanol	0.0086 (0.0047-0.016)*	14
140:1 LY235959/Butorphanol	0.012 (0.0057-0.026)*	10
Nalbuphine Alone	3.4 (2.1-5.5)	
0.46:1 LY235959/Nalbuphine	2.5 (1.8-3.6)	1.4
0.46:1 LY235959/Nalbuphine	1.5 (1.1-2.0)*	2.3
1.4:1 LY235959/Nalbuphine	1.1 (0.47-2.5)	3.1

Table 2.5 Assay ratios between the assays of schedule-controlled responding and thermal nociception. Assay ratios were defined for a LY235959/opioid mixture as [Dose Ratio (thermal nociception)] ÷ [Dose Ratio (schedule-controlled responding)].

Drug Mixture	Assay Ratio
LY235959 + Morphine	
1.3:1 LY235959/Morphine	6.9
3.9:1 LY235959/Morphine	2.2
12:1 LY235959/Morphine	1.6
LY235959 + Buprenorphine	
18:1 LY235959/Buprenorphine	4.1
54:1 LY235959/Buprenorphine	2.0
160:1 LY235959/Buprenorphine	2.1
LY235959 + Butorphanol	
15:1 LY235959/Butorphanol	3.7
45:1 LY235959/Butorphanol	7.4
140:1 LY235959/Butorphanol	1.2
LY235959 + Nalbuphine	
0.46:1 LY235959/Nalbuphine	0.74
0.46:1 LY235959/Nalbuphine	0.35
1.4:1 LY235959/Nalbuphine	0.10

Figure 2.1 Morphine, buprenorphine, butorphanol, nalbuphine, and LY235959 in the assay of schedule-controlled responding (left) and in the assay of thermal nociception (right). Abscissae, dose of drug in mg/kg. Ordinate, response rate as percentage of control rate of responding (left) or antinociception as percent maximum possible effect (right).

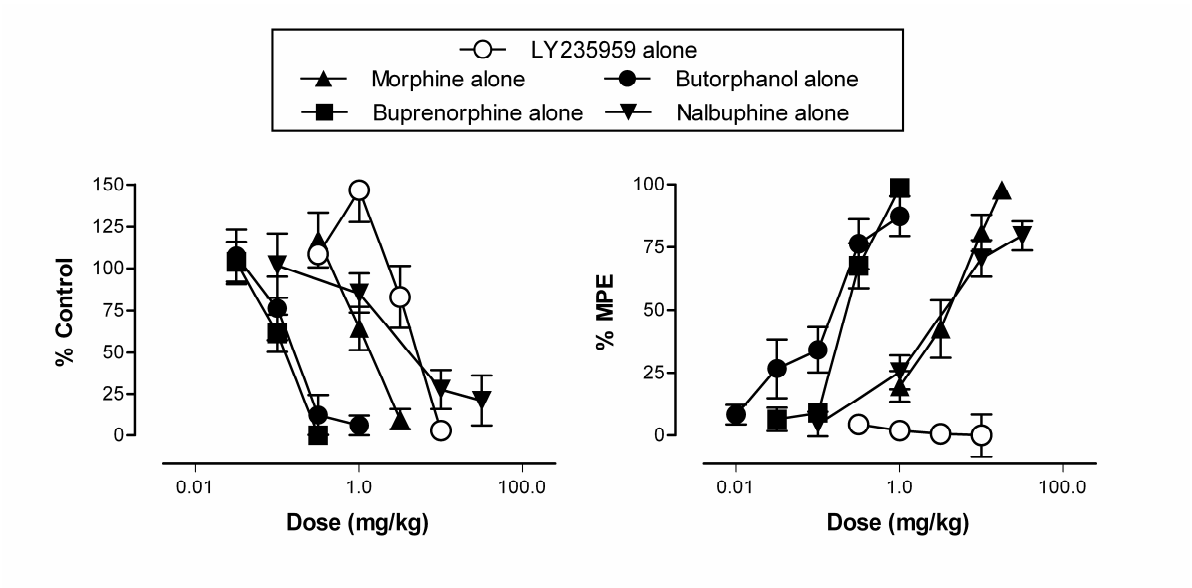


Figure 2.2 Morphine, buprenorphine, butorphanol, and nalbuphine alone and in combination with LY235959 in the assay of schedule-controlled responding. Top, dose-effect curves for opioid agonists alone and in combination with LY235959. Abscissae, dose of opioid in mg/kg. Ordinate, response rate as percentage of control. Bottom, isobolograms for LY235959/opioid mixtures. Abscissae, ED_{50} value for opioid in mg/kg. Ordinate, ED_{50} value for LY235959 in mg/kg.

*, Significantly different from additivity.

Figure 2.2

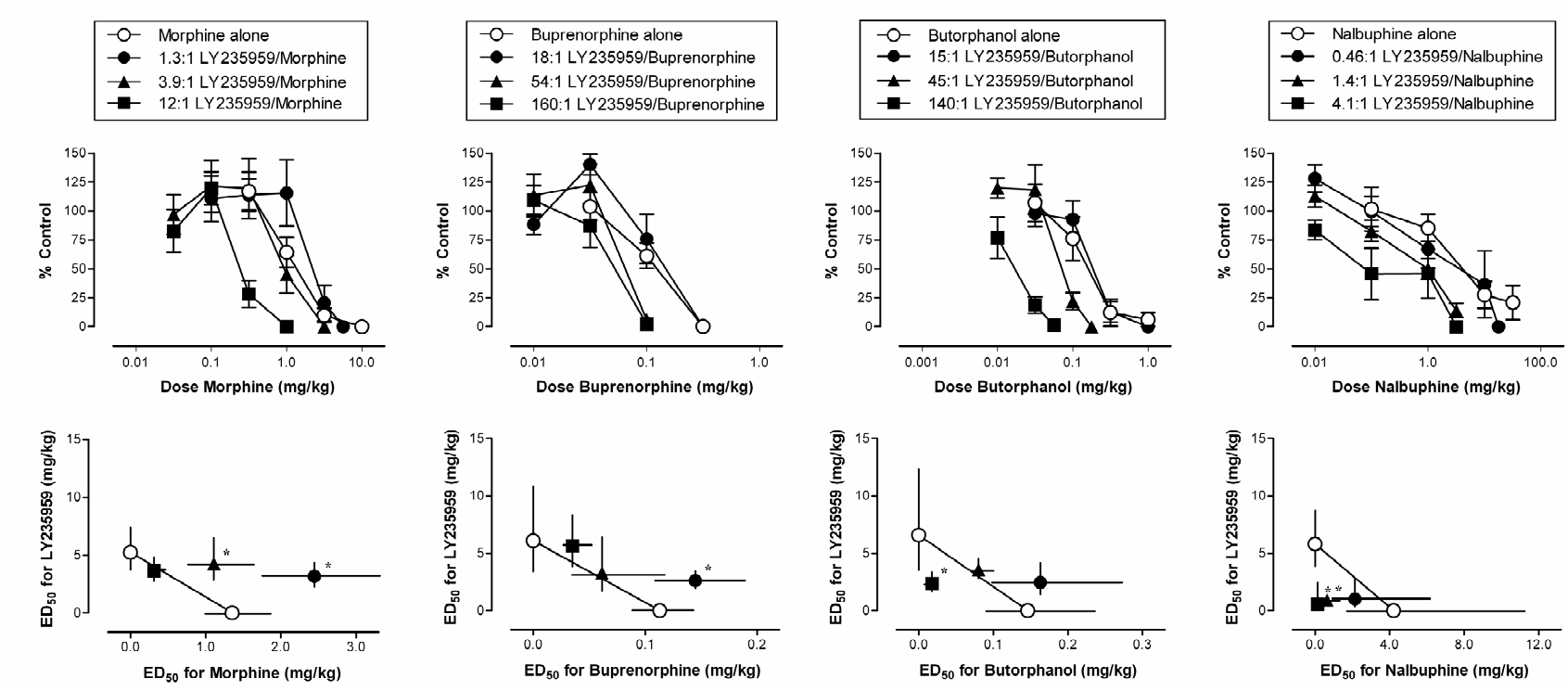
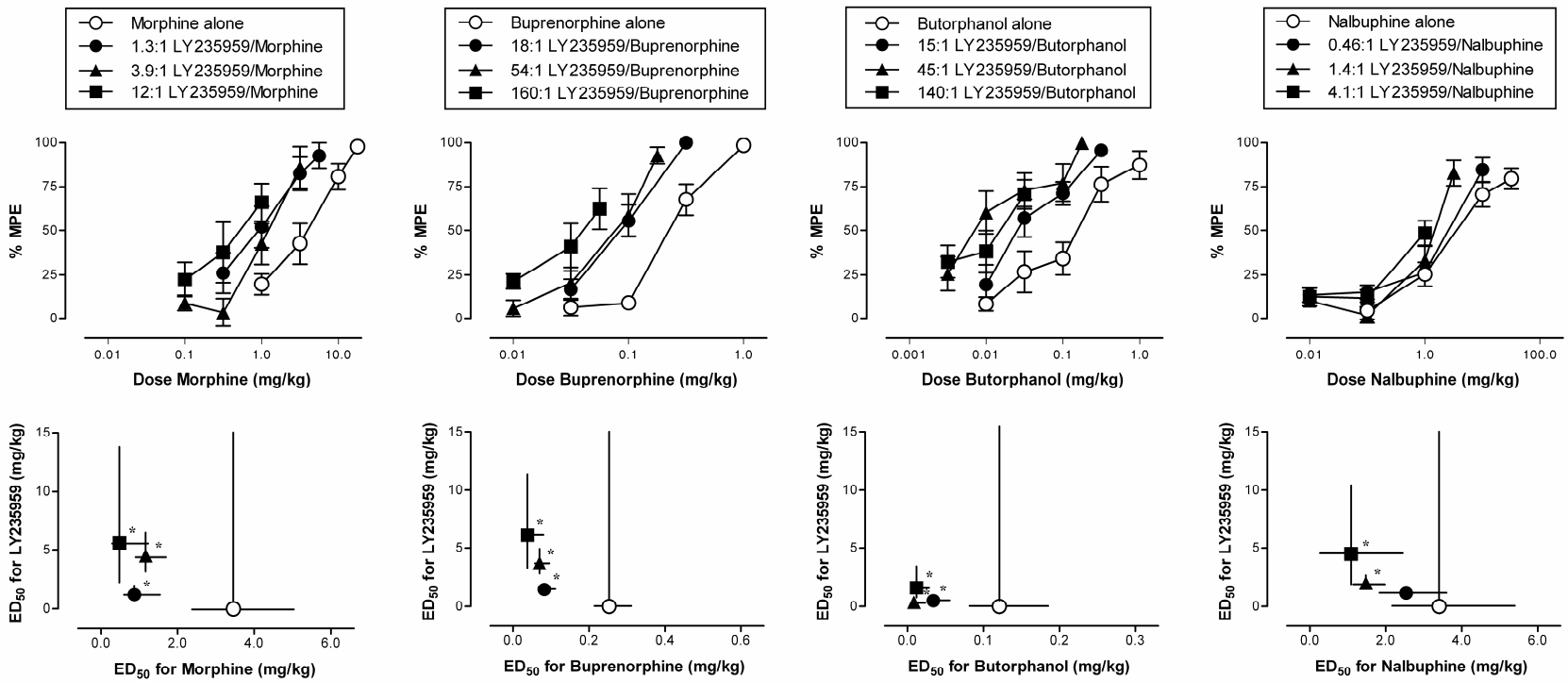

Morphine alone
1.3:1 LY235959/Morphine
3.9:1 LY235959/Morphine
12:1 LY235959/Morphine
Buprenorphine alone
18:1 LY235959/Buprenorphine
54:1 LY235959/Buprenorphine
160:1 LY235959/Buprenorphine
Butorphanol alone
15:1 LY235959/Butorphanol
45:1 LY235959/Butorphanol
140:1 LY235959/Butorphanol
Nalbuphine alone
0.46:1 LY235959/Nalbuphine
1.4:1 LY235959/Nalbuphine
4.1:1 LY235959/Nalbuphine
% Control
Dose Morphine (mg/kg)
Dose Buprenorphine (mg/kg)
Dose Butorphanol (mg/kg)
Dose Nalbuphine (mg/kg)
ED50 for LY235959 (mg/kg)
ED50 for Morphine (mg/kg)
ED50 for Buprenorphine (mg/kg)
ED50 for Butorphanol (mg/kg)
ED50 for Nalbuphine (mg/kg)

Figure 2.3 Morphine, buprenorphine, butorphanol, and nalbuphine alone and in combination with LY235959 in the assay of thermal nociception. Top, dose-effect curves for opioid agonists alone and in combination with LY235959. Abscissae, dose of opioid in mg/kg. Ordinate, antinociception as percent maximum possible effect. Bottom, isobolograms for LY235959/opioid mixtures. Abscissae, ED_{50} value for opioid in mg/kg. Ordinate, ED_{50} value for LY235959 in mg/kg.

*, Significantly different from additivity.

Figure 2.3


Morphine alone
1.3:1 LY235959/Morphine
3.9:1 LY235959/Morphine
12:1 LY235959/Morphine
Buprenorphine alone
18:1 LY235959/Buprenorphine
54:1 LY235959/Buprenorphine
160:1 LY235959/Buprenorphine
Butorphanol alone
15:1 LY235959/Butorphanol
45:1 LY235959/Butorphanol
140:1 LY235959/Butorphanol
Nalbuphine alone
0.46:1 LY235959/Nalbuphine
1.4:1 LY235959/Nalbuphine
4.1:1 LY235959/Nalbuphine
% MPE
Dose Morphine (mg/kg)
Dose Buprenorphine (mg/kg)
Dose Butorphanol (mg/kg)
Dose Nalbuphine (mg/kg)
ED50 for LY235959 (mg/kg)
ED50 for Morphine (mg/kg)
ED50 for Buprenorphine (mg/kg)
ED50 for Butorphanol (mg/kg)
ED50 for Nalbuphine (mg/kg)

CHAPTER 3

EXPERIMENT 2: MORPHINE IN COMBINATION WITH METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS ON SCHEDULE-CONTROLLED RESPONDING AND THERMAL NOCICEPTION

INTRODUCTION

Glutamatergic neurotransmission works by activating both ionotropic (iGlu) and G-protein-coupled metabotropic (mGlu) receptor subtypes, and is thought to play a modulatory role in the behavioral effects of morphine. To date, the most thoroughly investigated system of the glutamate receptor subtypes is the ionotropic *N*-Methyl-D-Aspartate (NMDA) receptor. Several lines of evidence suggest that pharmacological antagonism of the NMDA receptor blocks morphine-induced conditioned place preference (Papp et al., 2002), sensitization (Jeziorski et al., 1994), and physical dependence (Trujillo and Akil, 1991). NMDA receptor antagonists also attenuate the development of morphine tolerance (Trujillo and Akil, 1991; Kozela et al., 2003) and increase the acute antinociceptive effects of morphine (Nemmani et al. 2004; Fischer et al., 2005).

Pre-clinical data demonstrating that NMDA receptor antagonists increase the acute antinociceptive effects of morphine have led to the development of NMDA antagonist/morphine combinations for clinical use, albeit with mixed results (Bossard et al., 2002; Galer et al., 2005). The rationale for combination therapy is that NMDA antagonist/morphine mixtures may decrease morphine-induced side effects since increased

analgesia would be produced at a lower morphine dose. However, one disadvantage of this approach is the undesirable side effects associated with some NMDA antagonists (e.g. nausea, fatigue and dizziness, psychomimetic effects), most likely due to the ubiquitous involvement of NMDA receptors in excitatory neurotransmission throughout the central nervous system.

Interestingly, activation of postsynaptic mGlu receptors results in the potentiation of NMDA-mediated responses in dorsal horn neurons (Cerne and Randic, 1992; Kelso et al., 1992; Skeberdis et al., 2001), a region implicated in acute nociceptive neurotransmission. This potentiation likely modulates NMDA-mediated increases in morphine antinociception. Therefore, drugs that have antagonist action at postsynaptic mGlu receptors may provide an alternative to NMDA receptor antagonists in clinically therapeutic drug mixtures for the treatment of pain.

Recently, selective and bioavailable mGlu receptor antagonists have been synthesized. The present study was designed to assess the acute interactive effects of mGlu receptor antagonists and morphine on two behavioral endpoints. To assess the extent to which mGlu receptor antagonists selectively enhance morphine-induced antinociception, the rate-decreasing effects of mGlu antagonist/morphine mixtures were first examined in an assay of schedule-controlled responding maintained by liquid food. Second, the antinociceptive effects of mGlu antagonist/morphine mixtures were examined in an assay of thermal nociception. The interactive effects of mGlu antagonist/morphine mixtures were assessed using both graphical (isobolographic analysis; Loewe, 1953) and statistical (dose-addition analysis; Tallarida, 2000) approaches to distinguish effects that are additive from effects that are infra-additive or supra-additive.

To date, eight mGlu receptor subtypes have been identified, and have been divided into three groups: group I (mGlu1 and mGlu5), group II (mGlu2 and mGlu3), and group III (mGlu4, mGlu6, mGlu7, and mGlu8). The present study investigated the interactive effects of morphine in combination with antagonists selective for group I and group II mGlu receptors, as these receptor subtypes have been previously implicated in the behavioral effects of morphine on other endpoints (Popik and Wrobel, 2002; Kozela et al., 2003; Smith et al., 2004). The group I mGlu receptor antagonists used were (3,4-Dihydro-2H-pyrano[2,3-b]quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone (JNJ16259685) and 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) which display selectivity for mGlu1 and mGlu5 receptors, respectively (Gasparini et al., 1999; Lavreysen et al., 2004). The interactive effects of morphine and (2S)-2-Amino-2-[(1S,2S-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), a group II mGlu receptor antagonist (Kingston et al., 1998), were also assessed.

METHODS

Animals. Adult male C57BL/6 mice weighing between 22 and 30 g were purchased from Jackson Labs (Raleigh, NC). Upon arrival, mice were group housed in standard plexiglas cages in a colony room maintained on a 12-h light/dark cycle (lights on at 7:00 PM). All mice had continuous access to food and water throughout the study and were habituated to the colony room for 2 weeks prior to any experimental manipulation. Mice were also exposed to the testing environment and handled for two days prior to initiation of an experiment. All testing procedures were conducted between 11:00 AM and 3:00 PM. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and all testing adhered to the "Guide for the Care and Use of Laboratory Animals" (National Research Council, National Academy of Sciences, Washington, D.C., 1996).

Drugs. Morphine sulfate was provided by NIDA (Bethesda, MD). JNJ16259685, MPEP, and LY341495 were purchased from Tocris (Ellisville, MO). Morphine and LY341495 were dissolved in 0.9% phosphate buffered saline, and the pH of LY341495 solutions was adjusted to 7.0 with NaOH. JNJ16259685 and MPEP were dissolved in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin. All drugs were injected i.p. at a volume of 0.1 ml/10 g.

Schedule-Controlled Responding. Schedule-controlled responding was assessed in an experimental operant chamber (approximately 14 cm×14 cm×14 cm) equipped with a house light, ventilator fan, and two nosepoke holes (1.2 cm diameter) that were located on either side of a liquid dipper. The operant chamber was controlled by a MED-PC interface and an IBM compatible computer programmed with MED Associates software (MED Associates, St. Albans, VT).

Mice were trained under a multiple-cycle procedure conducted 5 days each week. Each training cycle consisted of a 25-min pretreatment period followed by a 5-min response period. During the pretreatment period, stimulus lights were not illuminated and responding had no scheduled consequences. During the response period, the right nosepoke was illuminated and mice could obtain up to 10 liquid food reinforcers (8 s access to 8 μl Ensure®) under a fixed ratio 3 schedule of food presentation. If all 10 reinforcers were earned before 5 min had elapsed, the light was turned off, and responding had no scheduled consequences for the remainder of the response period. The left nosepoke was inactive, and responding at this hole had no scheduled consequences. Training sessions consisted of 5 consecutive cycles, and testing began once response rates were stable throughout the session.

Test sessions replaced the last training session of each week if responding was stable throughout the preceding training sessions. For dose-effect curve determinations, test sessions were identical to training sessions except that cumulative doses of drug mixtures were administered i.p. at the start of each cycle (i.e., 30-min inter-injection interval), increasing in one-quarter or one-half log unit increments. For time course determinations, a single dose of a drug was administered i.p. at the start of the session, and 5-min response periods identical to those described above began at 10, 20, 40, 80, 160, and 240 min after the injection. Data are expressed as a percentage of control responding using the average rate of responding from the previous day as the control value (average of 5 cycles).

Thermal Nociception. Antinociception was assessed using a tail-flick analgesia meter (Columbus Instruments, Columbus, OH). For this procedure, the stimulus intensity was adjusted to provide baseline latencies between 3-5 s. The antinociceptive response was evaluated by recording the latency to flick the tail from the light source. Responses were

measured using a stopwatch to the nearest 0.1 s. A predetermined cutoff time of 10 s was defined as a maximal response and was employed to prevent tissue damage. Immediately following the termination of a trial, mice were removed from the apparatus and returned to the homecage. The latency to respond to the light source was measured twice at each determination, at least 30 s apart, with the light source focused 3 and 5 cm from the tip of the tail. These data were averaged to yield one value. Following baseline latency measurements, multiple 30 min cycles were conducted and drug mixtures were administered cumulatively. During dose-effect determinations, cumulative doses of drug mixtures were administered i.p. at the start of each cycle (i.e., 30-min inter injection interval), increasing in one-quarter or one-half log unit increments and antinociceptive measurements were determined during the last minute of each cycle. For time course determinations, a single injection of a drug or drug mixture was administered i.p., and antinociceptive measurements were assessed at 15, 30, 45, and 60 min. Latencies are expressed as a percentage of the maximal possible effect (%MPE) using the following formula: %MPE = [postdrug latency (seconds) - baseline latency (seconds)] / [cutoff time (10 s) - baseline latency (seconds)].

Dose-effect analysis. The dose of each drug mixture required to produce a 50% decrease in schedule-controlled responding or 50% maximum antinociceptive effect in the assay of thermal nociception was derived using log-linear interpolation by linear regression. Individual ED_{50} values were converted to their log values for calculation of means and 95% confidence limits and then converted back to linear values for presentation.

Isobolographic analysis. The effects of mGlu antagonist/morphine mixtures were assessed graphically with the use of isobolograms. In the present study, isobolograms were constructed by connecting the ED_{50} of morphine alone plotted on the abscissa with the ED_{50}

of the mGlu antagonist alone plotted on the ordinate to obtain an additivity line. The additivity line contains the loci of dose pairs that would produce an ED_{50} equal to the ED_{50} of morphine or an mGlu receptor antagonist alone if the combination is additive. Dose pairs that fall below the additivity line suggest an ED_{50} was reached with lesser quantities of the drugs, suggestive of supra-additivity. In contrast, experimental points representing dose pairs that fall above the line are suggestive of infra-additivity.

Isobolograms of dose pairs (a,b) were constructed based on the relative efficacy of morphine and an mGlu receptor antagonist. Morphine was fully efficacious in both assays, and was used as the reference drug. Therefore, the shape of the isobologram is dependent on the efficacy of the mGlu receptor antagonist under study from the equation

$$B = b + \frac{B}{\left(\frac{100}{A_{\max}}\right)\left(1 + \frac{A_c}{a}\right) - 1}$$

where B is the ED_{50} for morphine alone, $A_{\max}$ is related to the efficacy of the mGlu antagonist, and A_c is the dose of mGlu antagonist that attains half of $A_{\max}$ (Grabovsky and Tallarida, 2004). For the assay of schedule-controlled responding, in which all drugs were fully efficacious, the equation describing the isobologram is reduced to

$$B = b + \frac{B}{\left(\frac{A}{a}\right)} \quad \text{or} \quad \frac{a}{A} + \frac{b}{B} = 1 .$$

For the assay of thermal nociception, the mGlu antagonists JNJ16259685, MPEP, and LY341495 were ineffective when administered alone. Therefore, as the value of $A_{\max}$ approaches 0, the equation describing this isobologram reduces to

$$B = \lim_{A_{\max} \to 0} \left\{ b + \frac{B}{\left(\frac{100}{A_{\max}}\right)\left(1 + \frac{A_c}{a}\right) - 1} \right\} \quad \text{or} \quad B = b\,.$$

Dose-addition analysis. Drug interactions were statistically analyzed by comparing the experimentally determined ED_{50} values for each mixture (Z_{mix}) with predicted additive ED_{50} values (Z_{add}) as described by Tallarida (2000). Z_{mix} was defined as the total drug dose (i.e., dose morphine + dose mGlu receptor antagonist) that produced a 50% decrease in rates of responding (schedule-controlled responding) or a 50% maximum antinociceptive effect (thermal nociception).

For the assay of schedule-controlled responding, in which all drugs were equieffective, Z_{add} values were calculated individually for each mouse based on the ED_{50} values of each drug from the equation

$$Z_{add} = fA + (1 - f)B$$

where A is the ED_{50} for the mGlu receptor antagonist alone and B is the ED_{50} for morphine alone, and f is related to the proportion of the mGlu receptor antagonist in the mixture. The proportion of mGlu receptor antagonist (ρ_A) in each mixture is determined by the equation

$$\rho_A = \frac{fA}{fA + (1 - f)B}$$

and the proportion of morphine (ρ_B) in each mixture is determined by the equation

$$\rho_B = \frac{(1 - f)B}{fA + (1 - f)B}\,.$$

For the assay of thermal nociception, the mGlu antagonists JNJ16259685, MPEP, and LY341495 were ineffective when administered alone, and the hypothesis of additivity predicts that these drugs would not contribute to morphine's effects when administered in

combination (Tallarida, 2000). Therefore, Z_{add} is calculated based on the proportion of morphine in each particular mixture from the equation

$$Z_{add} = \frac{B}{\rho_B}.$$

Mean experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) for each mixture were compared with a t-test.

RESULTS

Schedule-controlled responding.

Morphine and mGlu antagonists alone. Figure 3.1 (top panel) shows that morphine, JNJ16259685, MPEP, and LY341495 produced dose-dependent decreases in the rate of responding. A statistical test for parallelism revealed that the morphine dose-effect curve was parallel to the dose-effect curves for JNJ16259685, MPEP, and LY341495 ($p < .05$). The mean ED_{50} values for each drug and the relative potencies for each mGlu antagonist in comparison to morphine are shown in Table 3.1. These relative potency values were used to determine relative proportions of the compounds used in subsequent studies assessing mGlu antagonist/morphine mixtures.

To confirm a similar duration of action, the rate-decreasing effects of morphine, JNJ16259685, MPEP, and LY341495 were assessed over time. As shown in Figure 3.2, each drug produced similar decreases in response rates (> 50% of control) for 40 min. Rates of responding gradually returned to near control rates at 160 min for morphine, JNJ16259685, and MPEP (91%, 88%, and 86% of control, respectively), and 240 min for LY341495 (81% of control). These data suggest that morphine, JNJ16259685, MPEP, and LY341495 produce similar peak effects and duration of action.

Morphine and group I mGlu antagonist mixtures. The rate-decreasing effects of morphine alone and in combination with JNJ16259685 and MPEP are shown in Figure 3.3. Each drug mixture produced dose-dependent decreases in response rates. Addition of JNJ16259685 produced leftward shifts in the morphine dose-effect curve, and the graphical analysis of these drug combinations is shown in Figure 3.3. Mixtures of JNJ16259685 and morphine produced additive effects across a range of proportions as these ED_{50} values fell

close to the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} = Z_{mix}$) (Table 3.2).

Addition of MPEP to morphine also produced leftward shifts in the morphine dose-effect curve and the magnitude of shift was correlated with the proportion of MPEP in the mixture. Figure 3.3 also shows the graphical analysis of these drug combinations. Mixtures of MPEP and morphine produced additive effects across a range of proportions as these ED_{50} values fell close to the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} = Z_{mix}$) (Table 3.2).

Morphine and group II mGlu antagonist mixtures. The rate-decreasing effects of morphine alone and in combination with LY341495 are shown in Figure 3.3. Each drug mixture produced dose-dependent decreases in response rates and addition of LY341495 to morphine produced leftward shifts in the morphine dose-effect curve. The magnitude of this shift was correlated with the proportion of LY341495 in the mixture. Figure 3.3 also shows the graphical analysis of these drug combinations. Mixtures of LY341495 and morphine produced additive effects across a range of proportions as these ED_{50} values fell close to the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} = Z_{mix}$) (Table 3.2).

Thermal nociception.

Morphine and mGlu antagonists alone. Figure 3.1 (bottom panel) shows the antinociceptive effects of morphine, JNJ16259685, MPEP, and LY341495. Morphine produced dose-dependent increases in latency to respond to the tail-flick apparatus, and this

ED_{50} value is shown in Table 3.1. JNJ16259685, MPEP, and LY341495 were without effect in this assay, therefore the relative potencies determined in the assay of schedule-controlled responding were used to determine the relative proportions of the compounds in each mixture.

Morphine and group I mGlu antagonist mixtures. The antinociceptive effects of morphine alone and in combination with JNJ16259685 and MPEP are shown in Figure 3.4. Each drug mixture produced dose-dependent increases in antinociception, and addition of JNJ16259685 produced leftward shifts in the morphine dose-effect curve. Graphical analysis of these drug combinations indicates that each JNJ16259685/morphine mixture produced supra-additive effects, as these ED_{50} values fell to the left of the line of additivity. Statistical comparison determined that the experimentally determined ED_{50} values (Z_{mix}) for these mixtures were significantly less than the predicted additive ED_{50} values (Z_{add}) (Table 3.3).

Addition of MPEP did not significantly shift the morphine dose-effect curve, as the ED_{50} values for MPEP/morphine mixtures were similar to the ED_{50} value of morphine alone. Graphical analysis of these drug combinations indicates that mixtures of MPEP and morphine produced additive effects across a range of proportions as these ED_{50} values fell close to the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} = Z_{mix}$) (Table 3.3).

The antinociceptive effects of morphine alone and in combination with JNJ16259685 and MPEP assessed over time are shown in Figure 3.5 (top panel). Morphine alone produced a moderate antinociceptive effect, with peak effects reached at 30 min post injection (13% maximum possible effect). Addition of JNJ16259685 produced a significant increase ($p <$

.05) in morphine-induced antinociception with peak effects reached at 45 min post injection (47% maximum possible effect). Coadministration of morphine and MPEP did not produce an antinociceptive effect that was significantly different from morphine alone at any time point.

Morphine and group II mGlu antagonist mixtures. The antinociceptive effects of morphine alone and in combination with LY341495 are shown in Figure 3.4. Each drug mixture produced dose-dependent increases in antinociception, and addition of LY341495 produced leftward shifts in the morphine dose-effect curve. The magnitudes of these shifts were correlated with the proportion of LY341495 in the mixture. Graphical analysis of these drug combinations indicates that the LY341495/morphine mixture with a lower proportion of LY341495 (i.e., 0.31:1 LY341495/morphine) produced additive effects, as these ED_{50} values fell close to line of additivity. In contrast, mixtures with a higher proportion of LY341495 (i.e., 0.93:1 and 2.8:1 LY341495/morphine) produced supra-additive effects, as these ED_{50} values fell to the left of the line of additivity. Statistical comparison of experimentally determined ED_{50} values (Z_{mix}) and predicted additive ED_{50} values (Z_{add}) confirmed these findings (i.e., $Z_{add} = Z_{mix}$ or $Z_{add} > Z_{mix}$) (Table 3.3).

The antinociceptive effects of morphine alone and in combination with LY341495 assessed over time are shown in Figure 3.5 (bottom panel). Morphine produced a moderate antinociceptive effect, with peak effects reached at 30 min post injection (13% maximum possible effect). Addition of LY341495 produced a significant increase ($p < .05$) in morphine-induced antinociception at 15, 30, and 45 min after drug administration. The peak effects of the LY341495/morphine mixture was reached at 30 min post injection (69% maximum possible effect).

DISCUSSION

The purpose of the present study was to assess the interactive effects of morphine and antagonists selective for group I and group II mGlu receptors on schedule-controlled responding and thermal nociception. The main finding from these experiments is that mGlu1 receptor antagonist/morphine mixtures and mGlu2/3 receptor antagonist/morphine mixtures produce a supra-additive effect on thermal nociception, whereas mGlu5 receptor antagonist/morphine mixtures produce an additive effect. Each mGlu antagonist/morphine mixture, however, produced an additive effect on schedule-controlled responding. Taken together, these data suggest that the interactive effects of mGlu antagonist/morphine mixtures depend on the mGlu receptor antagonist under study and the experimental endpoint.

Morphine and mGlu receptor antagonists administered alone.

In the assay of schedule-controlled responding, the μ-opioid receptor agonist morphine, the mGlu1 receptor antagonist JNJ16259685, the mGlu5 receptor antagonist MPEP, and the mGlu2/3 receptor antagonist LY341495 dose-dependently decreased rates of responding. In addition, the decreases in operant responding are time-dependent, with peak effects occurring at 10-40 min. Previous research suggests that MPEP also reduces operant responding at doses similar to those used in the present experiment (Varty et al., 2005), whereas the response rate altering effects of JNJ16259685 and LY341495 have not been assessed.

Previous research suggests that mGlu receptor antagonists produce antinociceptive effects in animal models of chronic pain (cf. Neugebauer, 2002). Reports on the acute antinociceptive effects of mGlu antagonists in assays of thermal nociception are limited, although at least one report suggests that mGlu antagonists with affinity for mGlu5 receptors do not modulate acute nociceptive processing (Walker et al., 2001). The present study further

suggests that mGlu antagonists selective for group I and group II receptor subtypes do not modulate acute nociceptive processing after systemic administration.

Morphine and group I mGlu receptor antagonist interactions.

The findings from the present study suggest that mixtures containing the mGlu1 receptor antagonist JNJ16259685 and morphine produce a supra-additive antinociceptive effect, while producing an additive effect on schedule-controlled responding. Although the present study is the first to demonstrate that an mGlu1 receptor antagonist potentiates morphine-induced antinociception, these effects have been evaluated in rats following antisense knockdown of mGlu1 receptors. In this study, knockdown of mGlu1 receptors increased the effectiveness of morphine in an animal model of neuropathic pain (Fundytus et al., 2001). The finding from the present study that JNJ16259685 increased morphine's antinociceptive effects corroborates this report. Taken together, these data suggest that mGlu1 receptors mediate the antinociceptive effects of morphine.

In contrast to the supra-additive effect of JNJ16259685/morphine mixtures on thermal nociception, mixtures containing the mGlu5 antagonist MPEP and morphine produced an additive effect on this endpoint. This agrees with and extends a study assessing single-dose combinations of MPEP and morphine (Kozela et al., 2003). Taken together, these data suggest that the antinociceptive effects of morphine may not be modulated by mGlu5 antagonists.

Both mGlu1 and mGlu5 receptors are expressed postsynaptically on dorsal horn neurons in the spinal cord (Vidnyanszky et al., 1994; Jia et al., 1999; Alvarez et al., 2000). Interestingly, these mGlu receptors are physically linked to NMDA receptors in this region (Naisbitt et al., 1999; Tu et al., 1999). Activation of mGlu1 and mGlu5 receptors results in

the potentiation of NMDA-mediated responses, through an activation of protein kinase C and a reduction of the voltage-dependent block of NMDA receptors by Mg^{2+} (Cerne and Randic, 1992; Kelso et al., 1992; Skeberdis et al., 2001). Increases in morphine-induced antinociception after NMDA antagonist administration have been demonstrated in assays of acute thermal nociception (Nemmani et al. 2004; Fischer et al., 2005). Therefore, the results from the present study suggest that inactivation of mGlu1 receptors, but not mGlu5 receptors, increases morphine-induced antinociception, most likely via a NMDA-dependent mechanism.

In view of the functional similarities of mGlu1 and mGlu5 receptors, the additive effects of MPEP/morphine mixtures on thermal nociception are surprising. Indeed, a growing body of experimental evidence indicates that MPEP modulates the effects of morphine on other endpoints (Popik and Wrobel, 2002; Kozela et al., 2003; Smith et al., 2004). Notably, as demonstrated in the current study, the behavioral effects of two drugs may depend on the experimental endpoint under study (Gessner and Cabana, 1970; Tallarida, 2000; Stevenson et al., 2003, 2005). In addition, these interactive effects may depend on factors including the relative proportion of the drugs in the mixture and the type of nociceptive assay used (Tallarida, 2000; Nemmani et al. 2004; Craft and Lee, 2005). Therefore, studies with MPEP/morphine mixtures in other proportions or in other assays of thermal nociception could yield results that are different from those reported here. Nevertheless, these data suggest that despite the functional similarities of mGlu1 and mGlu5 receptors, the manner in which they interact with morphine may be fundamentally different.

Morphine and group II mGlu receptor antagonist interactions.

The results from this study suggest that mixtures of morphine and the mGlu2/3 receptor antagonist LY341495 produce supra-additive effects in an acute model of thermal nociception after systemic administration. This finding agrees with a recent preliminary report by Yoon et al. (2006), which demonstrated that a LY341495/morphine mixture produces a supra-additive effect in the rat formalin test after intrathecal administration. The present study extends these findings to an acute model of nociception and to an assay of schedule-controlled responding, an endpoint where additive effects were observed.

The underlying mechanisms mediating the interactive effects of LY341495 and morphine in the assay of thermal nociception are not clear. Numerous reports have demonstrated that mGlu2/3 receptors are located presynaptically in numerous brain regions, and that drugs that act as *agonists* at these receptors decrease glutamate release and subsequent postsynaptic binding to both iGlu and mGlu receptor subtypes (Pin and Duvoisin, 1995). Indeed, some behavioral reports are consistent with this finding, suggesting that the mGlu2/3 agonist LY354740 attenuates behavioral signs of morphine tolerance and withdrawal in a similar manner as antagonists with affinity at the NMDA receptor or group I mGlu receptors (Klodzinska et al., 1999; Popik et al., 2000).

The specific function of mGlu2/3 receptors in the dorsal horn of the spinal cord, a region thought to mediate the behavioral responses in the assay of thermal nociception, is less clear. Both pre- and post-synaptic mGlu2/3 receptors have been identified in the dorsal horn (Carlton et al., 2001) and conflicting results are often reported from both *in vitro* and *in vivo* preparations. For example, mGlu2/3 agonists produce both facilitation and inhibition of neuronal activity (Bond and Lodge, 1995; Cao et al., 1995; King and Liu, 1996). In addition, both agonism and antagonism of mGlu2/3 receptors decrease pain related behaviors in

animal models of inflammation (Jones et al., 2005; Yoon et al., 2006). These results may reflect the complex interaction of pre- and post-synaptic mGlu2/3 receptors in the dorsal horn, and make the interpretation of mGlu2/3 antagonist/morphine interactions difficult. Therefore, further research is necessary to elucidate the mechanisms mediating the interactive effects of mGlu2/3 antagonist/morphine mixtures on nociceptive behavior.

Drug interactions across behavioral endpoints.

Previous research suggests that interactions between two drugs may depend on the experimental endpoint under study (Gessner and Cabana, 1970; Tallarida, 2000; Stevenson et al., 2003, 2005). For example, in Experiment 1, mixtures containing the NMDA antagonist LY235959 and morphine produced supra-additive effects on thermal nociception and additive or infra-additive effects on schedule-controlled responding. Similarly, in the present study, mixtures of morphine and the mGlu1 antagonist JNJ16259685 and mixtures of morphine and the mGlu2/3 antagonist LY341495 produced supra-additive effects in the assay of thermal nociception, while the same drug mixtures produced additive effects in the assay of schedule-controlled responding.

The behavioral selectivity of these interactions suggests that there is corresponding selectivity across brain regions mediating each behavior, rather than a general enhancement of all behavioral effects. The data from the present study suggest that it is possible to develop mGlu antagonist/morphine mixtures that interact in a supra-additive manner specifically at brain regions that mediate the targeted behavior of antinociception. Further research is necessary to expand the current findings across different measures of antinociception and on behavior maintained by other schedules of reinforcement. In addition, research assessing mGlu antagonist/morphine mixtures on other behavioral endpoints (e.g. respiratory

depression and/or drug self-administration) is necessary to predict further the clinical utility of these drug mixtures.

Table 3.1 ED_{50} values for morphine, JNJ16259685, MPEP, and LY341495 administered alone on schedule-controlled responding and thermal nociception.

Assay/Drug	ED_{50} value (± 95% CL)	Relative potency
Schedule-controlled responding		
Morphine	3.0 (1.9-5.0)	
JNJ16259685	2.6 (1.6-4.2)	0.87
MPEP	17 (10-28)	5.7
LY341495	2.8 (1.6-4.8)	0.93
Thermal nociception		
Morphine	5.6 (4.8-7.4)	
JNJ16259685	not determined	
MPEP	not determined	
LY341495	not determined	

Table 3.2 Predicted additive ED_{50} values (Z_{add}) and experimentally determined ED_{50} values (Z_{mix}) of morphine and mGlu receptor antagonist mixtures in the assay of schedule-controlled responding.

Drug Mixture	Z_{add} (± 95% CL)	Z_{mix} (± 95% CL)
JNJ16259685 + morphine		
0.29:1 JNJ16259685/morphine	1.7 (1.3-2.3)	3.1 (1.8-5.3)
0.87:1 JNJ16259685/morphine	3.5 (2.3-5.3)	3.1 (2.0-4.9)
2.6:1 JNJ16259685/morphine	3.1 (2.7-3.6)	3.0 (2.0-4.5)
MPEP + morphine		
1.9:1 MPEP/morphine	4.7 (2.1-11)	6.1 (3.9-9.4)
5.7:1 MPEP/morphine	6.9 (3.2-15)	9.5 (6.1-15)
17:1 MPEP/morphine	14 (5.5-35)	13 (8.2-21)
LY341495 + morphine		
0.31:1 LY341495/morphine	3.4 (2.0-5.9)	3.3 (2.2-4.9)
0.93:1 LY341495/morphine	2.9 (1.7-5.1)	3.3 (2.2-5.0)
2.8:1 LY341495/morphine	2.7 (1.3-5.6)	3.1 (2.0-4.9)

Table 3.3 Predicted additive ED_{50} values (Z_{add}) and experimentally determined ED_{50} values (Z_{mix}) of morphine and mGlu receptor antagonist mixtures in the assay of thermal nociception.

*, Z_{mix} significantly different from Z_{add} ($p < .05$).

[a], The highest dose tested (5.6 mg/kg morphine + 95.2 mg/kg MPEP) did not increase the latency to respond to the tail-flick apparatus to > 50% in three of eight mice. For analysis, a Z_{mix} of 100.8 was assigned to these mice.

Drug Mixture	Z_{add} (± 95% CL)	Z_{mix} (± 95% CL)
JNJ16259685 + morphine		
0.29:1 JNJ16259685/morphine	8.7 (6.2-12)	3.7 (2.5-5.4)*
0.87:1 JNJ16259685/morphine	13 (8.9-18)	2.6 (2.1-3.2)*
2.6:1 JNJ16259685/morphine	24 (17-34)	6.5 (4.1-10)*
MPEP + morphine		
1.9:1 MPEP/morphine	17 (12-26)	13 (10-16)
5.7:1 MPEP/morphine	40 (27-59)	34 (25-46)
17:1 MPEP/morphine	110 (71-160)	> 94 (84-100)[a]
LY341495 + morphine		
0.31:1 LY341495/morphine	7.0 (4.7-10)	5.0 (3.2-8.0)
0.93:1 LY341495/morphine	10 (7.0-15)	3.6 (2.1-6.3)*
2.8:1 LY341495/morphine	20 (14-30)	5.4 (3.5-8.3)*

Figure 3.1 Morphine, JNJ16259685, MPEP, and LY341495 in the assay of schedule-controlled responding (top) and in the assay of thermal nociception (bottom). Abscissae, dose of drug in mg/kg. Ordinate, response rate as percentage of control (top) or antinociception as percent maximum possible effect (bottom).

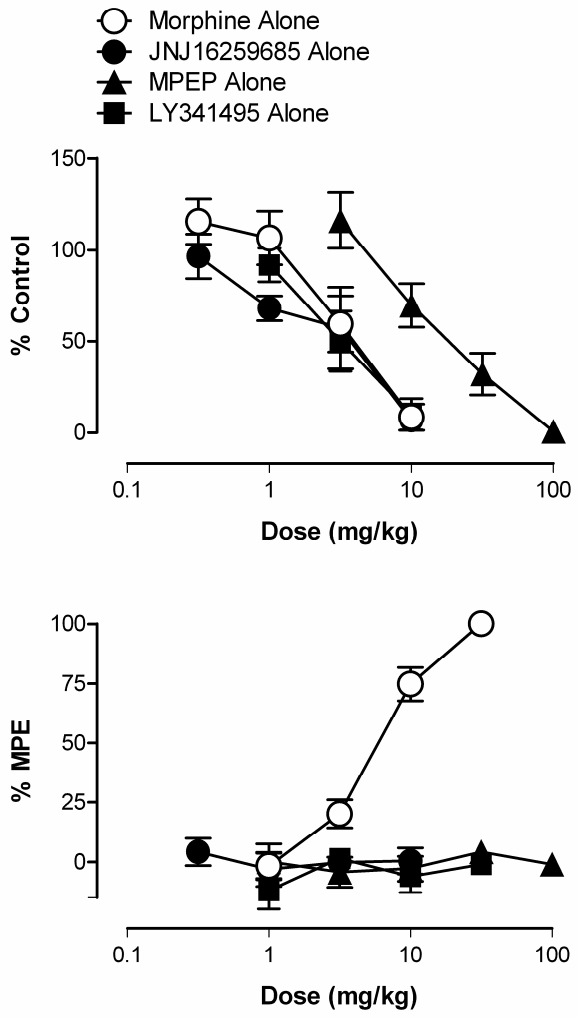


Figure 3.2 Morphine, JNJ16259685, MPEP, and LY341495 in the assay of schedule-controlled responding as assessed over time. Abscissae, time after drug administration. Ordinate, response rate as percentage of control rate of responding.

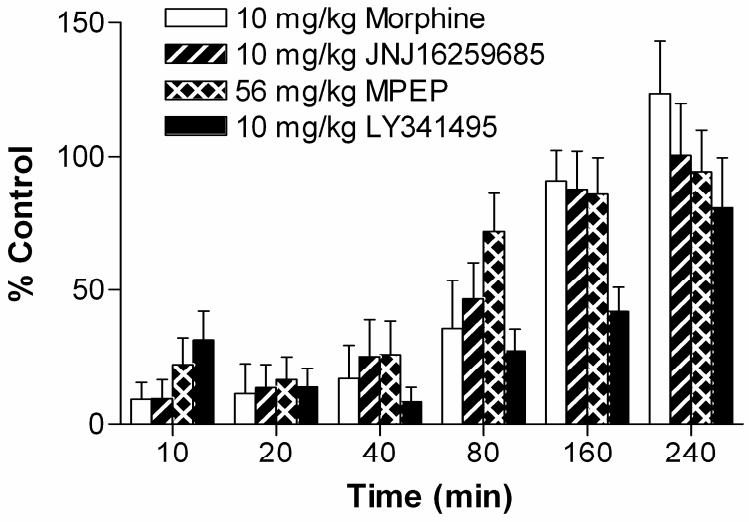


Figure 3.3 Morphine alone and in combination with JNJ16259685, MPEP, or LY341495 in the assay of schedule-controlled responding. Top, dose-effect curves for morphine alone and in combination with JNJ16259685, MPEP, or LY341495. Abscissae, dose of morphine in mg/kg. Ordinate, response rate as percentage of control. Bottom, isobolograms for mGlu antagonist/morphine mixtures. Abscissae, ED_{50} value for morphine in mg/kg. Ordinate, ED_{50} value for mGlu antagonist in mg/kg.

*, Significantly different from additivity.

Figure 3.3

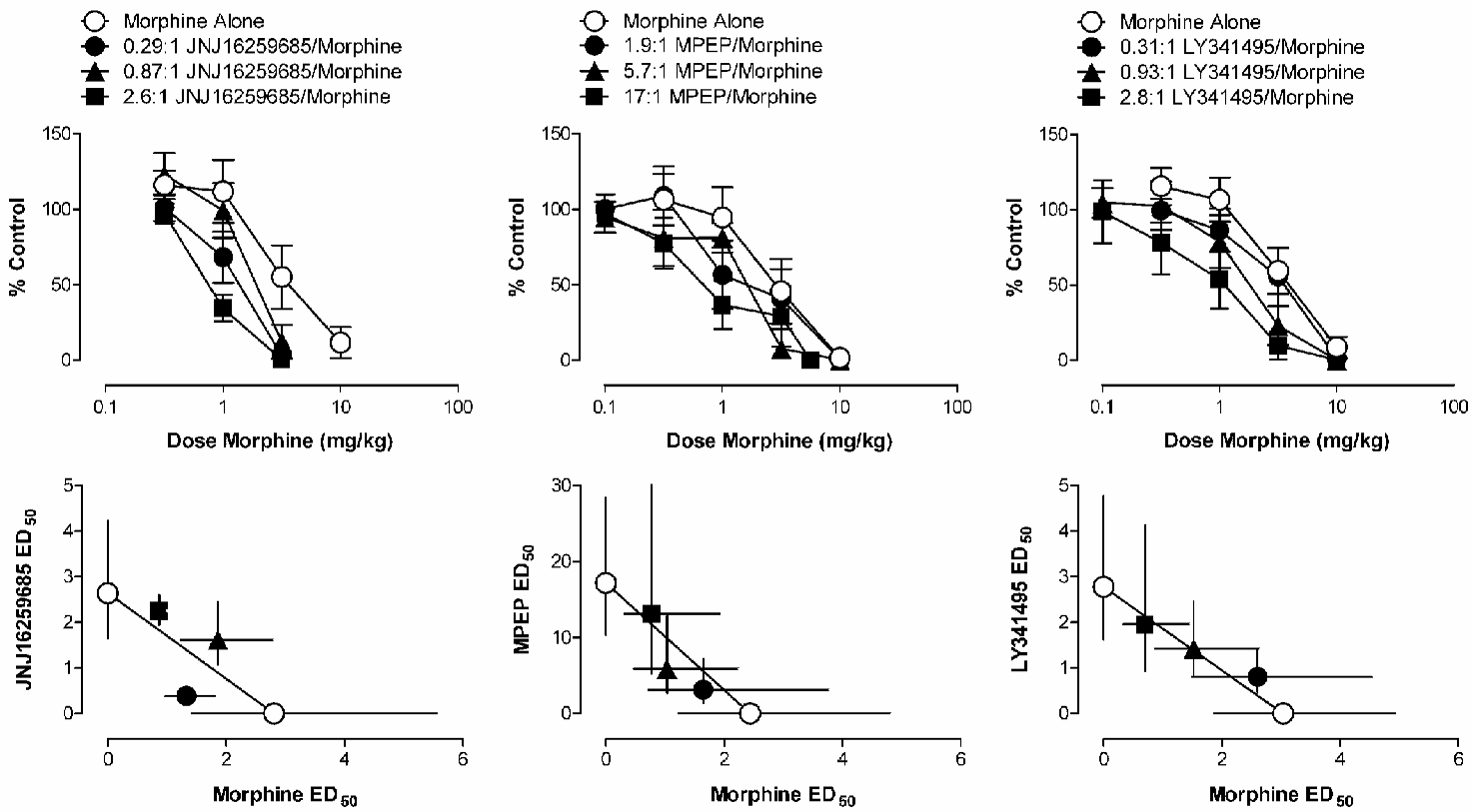

Morphine Alone
0.29:1 JNJ16259685/Morphine
0.87:1 JNJ16259685/Morphine
2.6:1 JNJ16259685/Morphine
Morphine Alone
1.9:1 MPEP/Morphine
5.7:1 MPEP/Morphine
17:1 MPEP/Morphine
Morphine Alone
0.31:1 LY341495/Morphine
0.93:1 LY341495/Morphine
2.8:1 LY341495/Morphine
% Control
Dose Morphine (mg/kg)
JNJ16259685 ED50
MPEP ED50
LY341495 ED50
Morphine ED50

Figure 3.4 Morphine alone and in combination with JNJ16259685, MPEP, or LY341495 in the assay of thermal nociception. Top, dose-effect curves for morphine alone and in combination with JNJ16259685, MPEP, or LY341495. Abscissae, dose of morphine in mg/kg. Ordinate, antinociception as percent maximum possible effect. Bottom, isobolograms for mGlu antagonist/morphine mixtures. Abscissae, ED_{50} value for morphine in mg/kg. Ordinate, ED_{50} value for mGlu antagonist in mg/kg.

*, Significantly different from additivity.

[>], The highest dose tested (5.6 mg/kg morphine + 95.2 mg/kg MPEP) did not increase the latency to respond to the tail-flick apparatus to > 50% in three of eight mice. For analysis, a morphine ED_{50} of 5.6 and a MPEP ED_{50} of 95.2 was assigned to these mice.

Figure 3.4

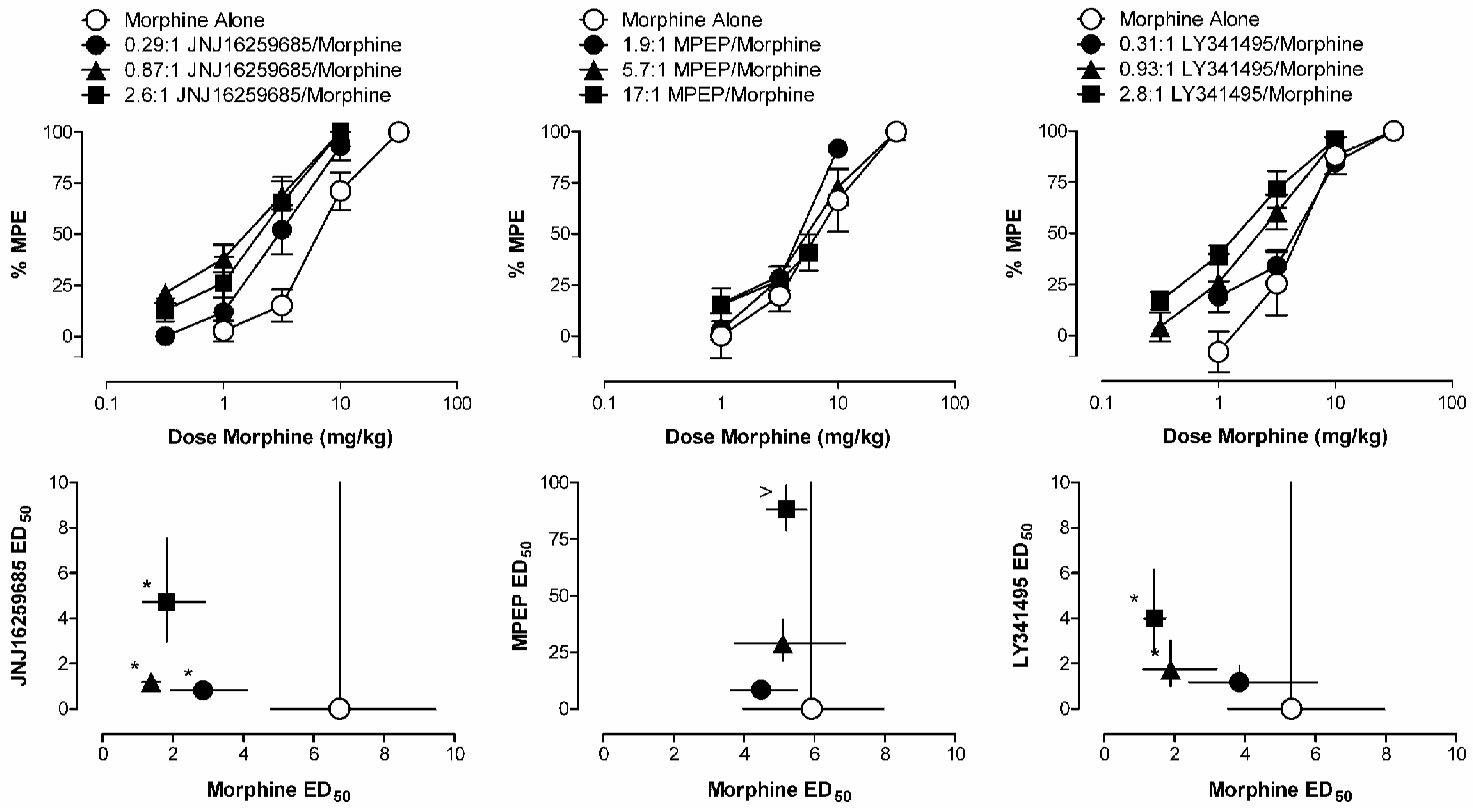
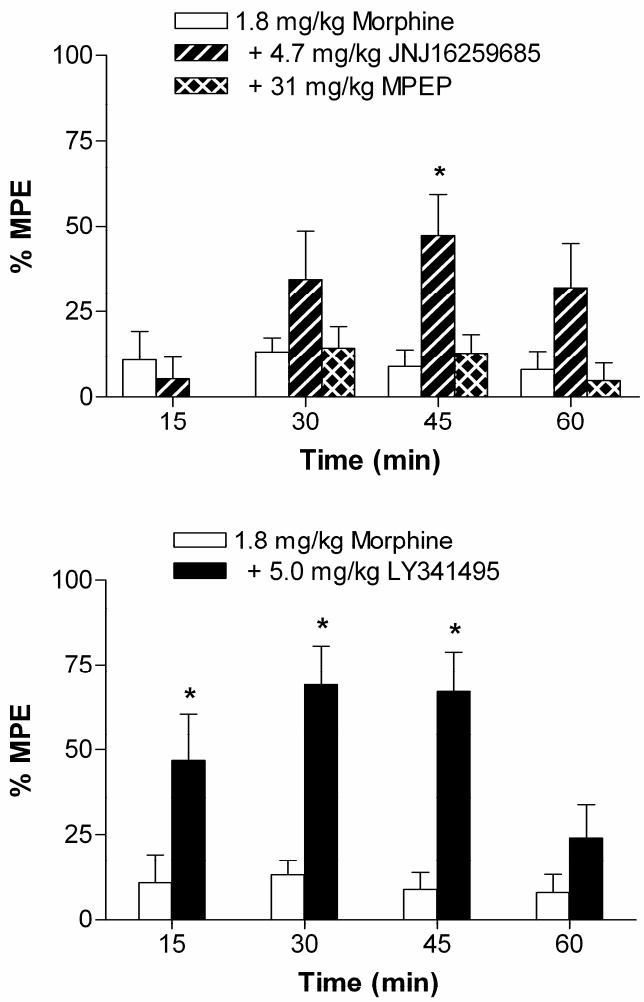

Morphine Alone
0.29:1 JNJ16259685/Morphine
0.87:1 JNJ16259685/Morphine
2.6:1 JNJ16259685/Morphine
% MPE
Dose Morphine (mg/kg)
JNJ16259685 ED50
Morphine ED50
Morphine Alone
1.9:1 MPEP/Morphine
5.7:1 MPEP/Morphine
17:1 MPEP/Morphine
% MPE
Dose Morphine (mg/kg)
MPEP ED50
Morphine ED50
Morphine Alone
0.31:1 LY341495/Morphine
0.93:1 LY341495/Morphine
2.8:1 LY341495/Morphine
% MPE
Dose Morphine (mg/kg)
LY341495 ED50
Morphine ED50

Figure 3.5 Morphine alone and in combination with JNJ16259685, MPEP, or LY341495 in the assay of thermal nociception as assessed over time. Abscissae, time after drug administration. Ordinate, antinociception as percent maximum possible effect.

*, Significantly different from morphine alone.



CHAPTER 4

EXPERIMENT 3: INCREASED EFFICACY OF μ-OPIOID AGONIST-INDUCED ANTINOCICEPTION BY METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS: COMPARISON WITH (-)-6-PHOSPHONOMETHYL-DECA-HYDROISOQUINOLINE-3-CARBOXYLIC ACID

INTRODUCTION

Opioid agonists with affinity at the μ-opioid receptor are commonly used for the treatment of various pain states. Behavioral evidence suggests that activation of subtype-specific glutamate receptors counteract the antinociceptive effects of μ-opioids. To date, the most thoroughly investigated system of these glutamate receptor subtypes is the ionotropic *N*-Methyl-D-Aspartate (NMDA) receptor. Evidence based on both pre-clinical and clinical studies suggest that pharmacological antagonists with affinity for the NMDA receptor increase the antinociceptive effects of μ-opioid receptor agonists across a range of testing conditions (Allen and Dykstra, 2001; Bossard et al., 2002; Nemmani et al., 2004; Fischer et al., 2005).

In addition to ionotropic receptors, endogenous glutamate exerts its action through a family of metabotropic glutamate (mGlu) receptors that activate various intracellular second messenger systems through G-proteins. To date, eight receptor subtypes have been identified and divided into three groups based on sequence similarity, pharmacology, and signal transduction mechanisms. Group I mGlu receptors include mGlu1 and mGlu5, and are

positively coupled to phospholipase C (PLC) through G_q. Group II mGlu receptors, including mGlu2 and mGlu3, and Group III mGlu receptors, including mGlu4, mGlu6, mGlu7, and mGlu8, are negatively coupled to adenylyl cyclase (AC) through G_i/G_o.

Recently, selective and bioavailable ligands with antagonist activity at mGlu receptors have been synthesized. New experimental evidence suggests that mGlu receptor antagonists with selectivity for either mGlu1 or mGlu2/3, but not mGlu5, also increase morphine-induced antinociception (Kozela et al., 2003; Yoon et al., 2006; results from Experiment 2). Although these data implicate mGlu receptors in opioid antinociception, the pharmacological basis of this interaction is poorly understood.

Numerous mechanisms may account for these findings, including interactive effects at a number of pharmacokinetic and pharmacodynamic loci (cf. Wessinger, 1986). For example, mGlu receptor antagonists may alter opioid absorption, distribution, metabolism, and/or excretion, thereby changing the effective concentration of the opioid at the μ-opioid receptor (e.g. Chen et al., 2005). In addition, mGlu receptor antagonists may directly modulate the μ-opioid receptor, thereby enhancing the affinity of the opioid for the receptor. Finally, the interactive effects of mGlu receptor antagonists and μ-opioid receptor agonists may result from alterations in the efficacy of the μ-opioid receptor through a change in G-protein mediated signaling cascades. Previous demonstrations that mGlu receptor antagonists produce leftward shifts in the morphine dose-effect curve do not discriminate between these possible mechanisms.

One research tool that can be used to delineate the mechanism by which mGlu receptor antagonists increase the antinociceptive effects of morphine is opioid intrinsic efficacy. Relative to high efficacy μ-opioid receptor agonists such as morphine, low-efficacy μ-opioid

receptor agonists are less effective in activating G-protein mediated processes and thus require a larger proportion of μ-opioid receptors to produce an effect. In assays of thermal nociception, the intensity of the nociceptive stimulus can be systematically manipulated to impose receptor requirements underlying a drug-induced effect. For example, under conditions of high stimulus intensity, a low-efficacy opioid agonist may produce only a partial effect if the number of receptors required to produce a maximum effect exceeds the number of opioid receptors available in the tissue mediating the effect. Under these conditions, an increase in opioid-induced antinociception by an inactive drug suggests that mGluR antagonists increase μ-opioid agonist efficacy..

The present study addresses the hypothesis that mGluR antagonists enhance opioid-induced antinociception through an increase in opioid receptor agonist efficacy. Morphine and the low-efficacy μ-opioid receptor agonists buprenorphine and dezocine are first assessed in a hot plate procedure under conditions of low (53°C) and high (56°C) stimulus intensity. Under conditions in which the low-efficacy μ-opioid receptor agonists produce sub-maximal effects, buprenorphine and dezocine are assessed after pretreatment with the mGlu1 receptor antagonist (3,4-Dihydro-2*H*-pyrano[2,3-b]quinolin-7-yl)-(*cis*-4-methoxycyclohexyl)-methanone (JNJ16259685), the mGlu5 receptor antagonist 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), and the mGlu2/3 receptor antagonist (2*S*)-2-Amino-2-[(1*S*,2*S*-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495). For comparison, the NMDA receptor antagonist (-)-6-phosphonomethyl-deca-hydroisoquinoline-3-carboxylic acid (LY235959) is also assessed as a pretreatment to morphine and the low-efficacy μ-opioid receptor agonists.

METHODS

Animals. Adult male C57BL/6 mice 10 weeks of age were purchased from Jackson Laboratory (Raleigh, NC). Upon arrival, mice were group housed in standard plexiglas cages in a colony room maintained on a 12-h light/dark cycle (lights on at 7:00 PM). All mice had continuous access to food and water throughout the study and were habituated to the colony room environment for 2 weeks prior to any experimental manipulation. Mice were also exposed to the testing environment and handled for two days prior to initiation of an experiment. All testing procedures were conducted between 11:00 AM and 3:00 PM. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill and all testing adhered to the "Guide for the Care and Use of Laboratory Animals" (National Research Council, National Academy of Sciences, Washington, D.C., 1996).

Drugs. Morphine sulfate and buprenorphine hydrochloride were provided by NIDA (Bethesda, MD), dezocine hydrochloride by AstraZeneca (Wilmington, DE), and LY235959 by Lilly Research Laboratories (Indianapolis, IN). JNJ16259685, MPEP, and LY341495 were purchased from Tocris (Ellisville, MO). Morphine, LY235959, and LY341495 were dissolved in 0.9% phosphate buffered saline, and the pH of LY341495 solutions was adjusted to 7.0 with NaOH. JNJ16259685 and MPEP were dissolved in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin. All drugs were injected i.p. at a volume of 0.1 ml/10 g.

Thermal nociception. Antinociception was assessed using a hot plate analgesia meter (25.3 × 25.3 cm) (Columbus Instruments, Columbus, OH) maintained at either 53 ± 0.1°C or 56 ± 0.1°C. The antinociceptive response was evaluated by recording the latency to lick or shuffle the hind paw(s), and/or to jump from the hot plate surface. A stopwatch measured

responses to the nearest 0.1 s. A predetermined cutoff time of 20 s was defined as a maximal response and was employed to prevent tissue damage. Immediately following the termination of a trial, mice were removed from the hot plate surface and returned to the homecage. The antinociceptive response was measured twice at 30 and 15 min prior to the beginning of drug administration. These data were averaged to yield one baseline value. After baseline measurements, saline or a glutamate receptor antagonist (i.p.) was administered 30 min prior to administration of an opioid agonist (s.c.). Antinociceptive responses were recorded at 15, 30, 45, 60, 90, and 120 min following administration of the second drug. Latencies obtained following drug administration represent one trial and are expressed as a percentage of the maximal possible effect (%MPE) from the following formula: %MPE = [postdrug latency (seconds) - baseline latency (seconds)] / [20 - baseline latency (seconds)].

Data analysis. Data are expressed as the mean (± S.E.M.) antinociceptive response. Dose-effect curves were constructed from data obtained 30 min following administration of an opioid agonist. If an opioid was fully efficacious, the dose required to produce a 50% (ED_{50}) maximal antinociceptive effect was derived using log-linear interpolation. Statistical analyses for buprenorphine and dezocine dose-effect curves in combination with a glutamate receptor antagonist were conducted using a two-factor analysis of variance (ANOVA). Fisher's protected least significant difference (PLSD) was then conducted to determine specific opioid dose combinations that were significantly different from baseline. All statistical analyses were conducted with a level of significance set at $p < .05$.

RESULTS

Figure 4.1 shows the antinociceptive effects of buprenorphine, dezocine, and morphine under conditions of low (53°C) and high (56°C) stimulus intensity. At 53°C, each drug produced dose-dependent increases in antinociception resulting in ED_{50} values (± 95% confidence limits) of 0.17 (0.12-0.23) for buprenorphine, 0.33 (0.28-0.41) for dezocine, and 3.4 (2.7-4.4) for morphine. At 56°C, only morphine produced dose-dependent antinociception, resulting in an ED_{50} value of 7.7 (6.2-9.6). At this stimulus intensity, buprenorphine and dezocine produced sub-maximal antinociception (i.e. < 50% MPE), and an ED_{50} value could not be determined. To confirm a decrease in efficacy of buprenorphine and dezocine, the highest doses tested were assessed in combination with a dose of morphine (32 mg/kg) that produced 100% MPE (data not shown). When administered in combination with morphine, 3.2 mg/kg buprenorphine significantly decreased its antinociceptive effects, resulting in a MPE (± 95% confidence limits) of 36% (21%-50%). In addition, 10 mg/kg dezocine significantly decreased the antinociceptive effects of morphine, resulting in a MPE of 38% (21%-55%).

The effects of glutamate receptor antagonists on morphine antinociception as assessed in a tail-flick procedure have been previously described in Experiments 1 and 2, and these previous findings were replicated in the current experiment on the hot plate. Similar to the previous reports, morphine's antinociceptive potency was increased when administered in combination with the mGlu1 receptor antagonist JNJ16259685, the mGlu2/3 receptor antagonist LY341495, and the NMDA receptor antagonist LY235959 as evidenced by significant leftward shifts in the morphine dose-effect curve. The antinociceptive potency of morphine was not increased when administered in combination with the mGlu5 receptor

antagonist MPEP. The resulting ED_{50} values for morphine in combination with JNJ16259685, MPEP, LY341495, and LY235959 are shown in Table 4.1.

Figure 4.2 shows the antinociceptive effects of buprenorphine and dezocine, alone and in combination with the mGlu1 antagonist JNJ16259685 (1.0-3.2 mg/kg). JNJ16259685 did not produce an antinociceptive effect when administered alone (points above "S"). Pretreatment with JNJ16259685 significantly shifted the dose-effect cures for buprenorphine ($F_{14,119}$ = 15.82, P < .001) and dezocine ($F_{11,95}$ = 20.84, P < .001) upward. PLSD analysis revealed that both 1.0 and 3.2 mg/kg JNJ16259685 significantly increased antinociception at 0.32-3.2 mg/kg buprenorphine and at 3.2-10 mg/kg of dezocine. When assessed over time, JNJ16259685 significantly increased the antinociceptive effects of both 1.0 mg/kg buprenorphine ($F_{1,21}$ = 16.86, P < .001) and 3.2 mg/kg dezocine ($F_{1,21}$ = 25.83, P < .001) during the 120 min test period. PLSD analysis revealed that 1.0 mg/kg JNJ16259685 significantly increased antinociception at time points 15-30 min for buprenorphine, and that 3.2 mg/kg JNJ16259685 significantly increased the antinociceptive effects over the 120 min timecourse for buprenorphine and dezocine.

The antinociceptive effects of buprenorphine and dezocine were also assessed after pretreatment with the mGlu5 receptor antagonist MPEP, and are shown in Figure 4.3. Administration of MPEP (1.0-3.2 mg/kg) did not produce an antinociceptive effect when administered alone. When administered as pretreatment to 1.0 mg/kg buprenorphine and 3.2 mg/kg dezocine, MPEP did not increase antinociception relative to buprenorphine or dezocine administered alone at any time point across the 120 min timecourse.

Figure 4.4 shows the antinociceptive effects of buprenorphine and dezocine, alone and after pretreatment with the mGlu2/3 antagonist LY341495. When administered alone,

LY341495 (1.0-3.2 mg/kg) did not produce an antinociceptive effect. Pretreatment with LY341495 prior to administering buprenorphine and dezocine significantly shifted the dose-effect curve for both drugs upward (buprenorphine, $F_{14,119} = 15.57$, $P < .001$; dezocine, $F_{11,95} = 14.81$, $P < .001$). PLSD analysis revealed that 1.0 mg/kg LY341495 significantly increased antinociception at 0.1-1.0 mg/kg buprenorphine and at 10 mg/kg dezocine. Analysis also revealed that 3.2 mg/kg LY341495 increased the effects of 0.1-3.2 mg/kg buprenorphine and 3.2-10 mg/kg dezocine. When assessed over time, LY341495 significantly increased the antinociceptive effects of both 1.0 mg/kg buprenorphine ($F_{1,21} = 17.38$, $P < .001$) and 3.2 mg/kg dezocine ($F_{1,21} = 23.22$, $P < .001$) during the 120 min test period. PLSD analysis revealed that 3.2 mg/kg LY341495 significantly increased antinociception across the 120 min timecourse for buprenorphine and dezocine.

Figure 4.5 shows the antinociceptive effects of buprenorphine and dezocine, both alone and in combination with the NMDA receptor antagonist LY235959 (0.32-1.0 mg/kg). When administered alone, LY235959 did not produce an antinociceptive effect. When administered as a pretreatment to buprenorphine and dezocine, LY235959 significantly shifted the dose-effect curve for both drugs upward (buprenorphine, $F_{14, 118} = 19.77$, $P < .001$; dezocine, $F_{11,95} = 16.55$, $P < .001$). PLSD analysis revealed that 0.32 mg/kg LY235959 significantly increased the antinociceptive effect of 0.32-3.2 mg/kg buprenorphine and 3.2-10 mg/kg dezocine. Analysis also revealed that 1.0 mg/kg LY235959 increased buprenorphine's effects at 0.1-3.2 mg/kg and dezocine's effects at 1.0-10 mg/kg. When assessed over time, LY235959 significantly increased the antinociceptive effects of both 1.0 mg/kg buprenorphine ($F_{1,20} = 14.48$, $P < .001$) and 3.2 mg/kg dezocine ($F_{1,21} = 15.51$, $P < .001$) during the 120 min test period. PLSD analysis revealed that 1.0 mg/kg LY235959

significantly increased antinociception at time points 30-90 min for buprenorphine and 15-90 min for dezocine.

DISCUSSION

The present series of studies examined the interactive effects of opioids and glutamate receptor antagonists that vary in selectivity. In agreement with previous reports (Allen and Dykstra, 2001; Fischer et al., 2005; Yoon et al., 2006; results from Experiments 1 and 2), the mGlu1 receptor antagonist JNJ16259685, the mGlu2/3 receptor antagonist LY341495, and the NMDA antagonist LY235959 increased the antinociceptive *potency* of morphine as evidenced by ~ 3-fold leftward shifts in the morphine dose-effect curve (Table 4.1). This effect was dependent on the pharmacological selectivity of the glutamate receptor antagonist under study since JNJ16259685, LY341495, and LY235959 shifted the morphine dose-effect curve to the left, but the mGlu5 receptor antagonist MPEP did not. Together, these data confirm previous investigations (Allen and Dykstra, 2001; Kozela et al., 2003; Fischer et al., 2005; Yoon et al., 2006; results from Experiments 1 and 2) indicating that JNJ16259685, LY341495, and LY235959, but not MPEP, increase the antinociceptive potency of morphine

The initial purpose of the present study was to develop behavioral conditions under which to examine the effects of mGlu receptor antagonists on the *efficacy* of μ-opioid receptor agonists. Towards this end, the effects of them μ-opioid receptor agonists buprenorphine, dezocine, and morphine were examined under conditions of relatively low (53°C) and high (56°C) nociceptive stimulus intensities. Morphine produced dose-dependent increases in antinociception, and was fully efficacious under both conditions. In contrast, the efficacy of both buprenorphine and dezocine was dependent on the intensity of the nociceptive stimulus. Under conditions of low stimulus intensity, both buprenorphine and dezocine produced dose-dependent antinociceptive effects, and were fully efficacious. Under conditions of high stimulus intensity, however, increasing doses of buprenorphine and

dezocine produced only low levels of antinociception and in no instance did the maximal effect of these drugs exceed 50% MPE.

Buprenorphine and dezocine are lower in efficacy relative to morphine (Chen et al., 1992; Toll, 1995; Gharagozlou et al., 2003). By definition, low-efficacy μ-opioid receptor agonists activate G-protein mediated processes less efficiently and require a larger proportion of receptors to produce an effect. The findings from the present study are in agreement with assumptions derived from receptor theory. Under conditions of low stimulus intensity, the number of μ-opioid receptors required to produce a maximal effect is relatively low and a maximum effect is attainable by both high and low-efficacy agonists (cf. Morgan et al., 1999). In contrast, when high stimulus intensities are used, the proportion of μ-opioid receptors required to produce a maximal effect is increased, and the maximal effect attainable by a μ-opioid receptor agonist is dependent on the intrinsic efficacy of the μ-opioid receptor agonist under study (Morgan et al., 1999). In agreement with these assumptions, buprenorphine and dezocine produced only a partial antinociceptive effect under conditions of high stimulus intensity.

The main purpose of the present study was to test the hypothesis that mGlu receptor antagonists increase opioid antinociception through an alteration in the efficacy of the μ-opioid receptor, and to compare these results with data obtained with an NMDA receptor antagonist. If the aforementioned increases in opioid potency were due to mechanisms other than alterations in efficacy and subsequent G-protein mediated signaling cascades, it would be expected that pretreatment with a glutamate receptor antagonist would result in leftward shifts in the buprenorphine and dezocine dose-effect curves, without an increase in their maximal effects. The main finding from these experiments is that, similar to the NMDA

antagonist LY235959, the mGlu1 receptor antagonist JNJ16259685 and the mGlu2/3 receptor antagonist LY341495, but not the mGlu5 receptor antagonist MPEP, increased the efficacy of buprenorphine and dezocine. These data suggest that, similar to an NMDA receptor antagonist, mGlu1 and mGlu2/3 antagonists increase opioid-induced antinociception through an alteration in the efficacy of the μ-opioid receptor.

Although speculative, the interactive effects of JNJ16259685 and μ-opioid receptor agonists can be conceptualized through receptor-mediated processes. Both μ-opioid receptors and mGlu1 receptors are expressed postsynaptically on dorsal horn neurons in the spinal cord (Jia et al., 1999; Alvarez et al., 2000), a region thought to mediate nociceptive transmission. In this region, mGlu1 receptors are physically linked to NMDA receptors through postsynaptic density proteins (Naisbitt et al., 1999; Tu et al., 1999). Activation of mGlu1 receptors in the dorsal horn results in the potentiation of NMDA-mediated responses, through an activation of protein kinase C and a reduction of the voltage-dependent block of NMDA receptors by Mg^{2+} (Cerne and Randic, 1992; Kelso et al., 1992; Skeberdis et al., 2001). In view of the observed increases in μ-opioid receptor agonist-induced antinociception after NMDA antagonist administration, it is possible that the increases after administration of an mGlu1 antagonist results from functionally similar processes.

In addition to the mGlu1 antagonist JNJ16259685, the mGlu2/3 receptor antagonist LY341495 increased the efficacy of buprenorphine and dezocine; however, the underlying mechanisms mediating this interaction are less clear. Numerous reports have demonstrated that mGlu2 and mGlu3 receptors are located presynaptically in numerous brain regions, although both pre- and post-synaptic receptors have been identified in the dorsal horn (Carlton et al., 2001). As a result, conflicting results are often reported from both *in vitro* and

in vivo preparations. For example, mGlu2/3 agonists produce both facilitation and inhibition of neuronal activity (Bond and Lodge, 1995; Cao et al., 1995; King and Liu, 1996), and both agonism and antagonism of mGlu2 and mGlu3 receptors decrease pain related behaviors in animal models of inflammation (Jones et al., 2005; Yoon et al., 2006). These results may reflect the complex interaction of pre- and post-synaptic receptors in the dorsal horn, and make it difficult to interpret the mGluR2/3-mediated increases in opioid efficacy observed in the current study. Nevertheless, the data from the current study suggests that antagonism of mGlu2 and mGlu3 receptors increase the efficacy of μ-opioid receptor agonists through alterations in the efficacy of the μ-opioid receptor.

Although the intention of assessing the antinociceptive effects of buprenorphine and dezocine under conditions of high stimulus intensity in the present study was to enhance the understanding of the pharmacological nature of mGlu receptor/opioid interactions, the findings from these experiments may also have important clinical implications. Although low-efficacy opioid agonists such as buprenorphine and dezocine are less effective than morphine, they are often characterized as having a lower potential for abuse and an improved side-effect profile (Hoskin and Hanks, 1991; Preston and Jasinski, 1991). If glutamate receptor antagonists increase the functional efficacy of these drugs in assays of nociception, without increasing effects unrelated to antinociception (cf. Experiment 2), combination treatment might be useful for the management of various pain states. Clearly, the clinical utility of these drug combinations will rely on behavioral assessment on other endpoints, such as models of abuse potential, physical dependence, and other off-target effects.

Table 4.1 ED_{50} values and dose ratios for morphine alone and in combination with JNJ16259685, MPEP, LY341495, and LY235959 on thermal nociception at 56°C. *, Significantly different from morphine alone.

	ED_{50} (± 95% CL)	Dose Ratio
Morphine Alone	7.7 (6.2-9.6)	
+ 3.2 mg/kg JNJ16259685	2.2 (1.7-2.8)*	3.5
+ 3.2 mg/kg MPEP	9.0 (7.2-11)	0.86
+ 3.2 mg/kg LY341495	2.7 (2.1-3.4)*	2.9
+ 1.0 mg/kg LY235959	2.2 (1.5-3.3)*	3.5

Figure 4.1 Buprenorphine, dezocine, and morphine on thermal nociception. Top, dose-effect curves for buprenorphine, dezocine, and morphine at 53°C. Bottom, dose-effect curves for buprenorphine, dezocine, and morphine at 56°C. Abscissa, dose of opioid in mg/kg. Ordinate, antinociception as percent maximum possible effect.

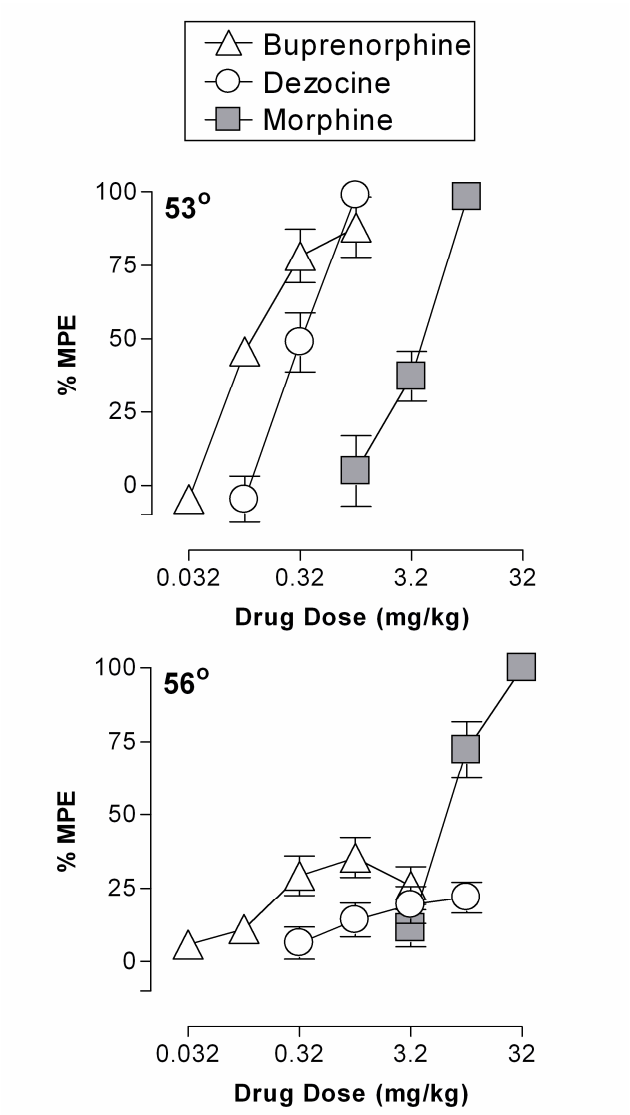


Figure 4.2 Buprenorphine and dezocine in combination with JNJ16259685 on thermal nociception at 56°C. Top, dose effect curves for buprenorphine (left) and dezocine (right) alone and in combination with JNJ16259685. Abscissa, dose of buprenorphine or dezocine in mg/kg. Ordinate, antinociception as percent maximum possible effect. Bottom, timecourse of 1.0 mg/kg buprenorphine (left) and 3.2 mg/kg dezocine (right) alone and in combination with JNJ16259685. Abscissa, time after administration of buprenorphine or dezocine. Ordinate, antinociception as percent maximum possible effect.

*, Significantly different from buprenorphine or dezocine alone.

Figure 4.2

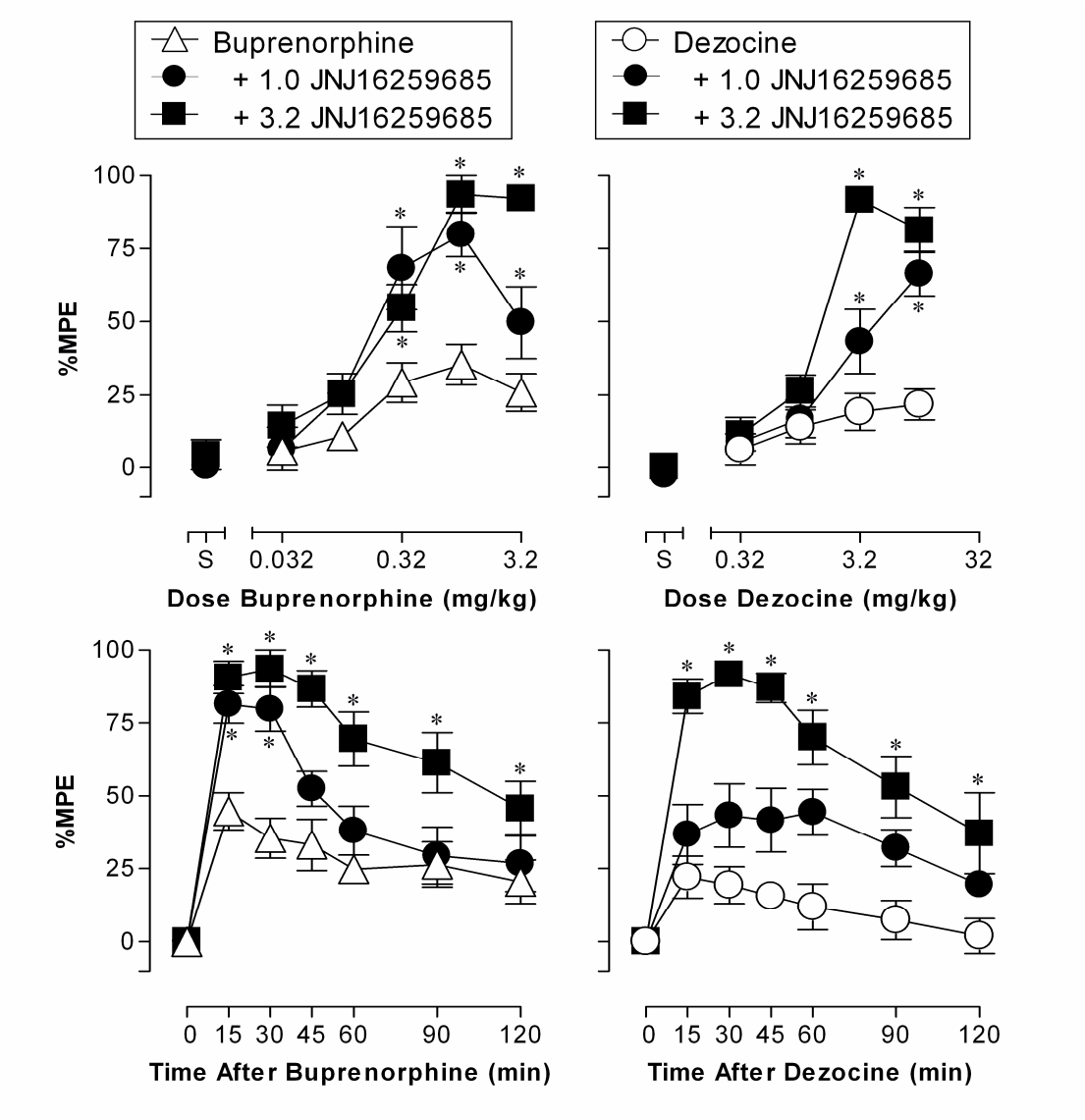

Buprenorphine
+ 1.0 JNJ16259685
+ 3.2 JNJ16259685
Dezocine
+ 1.0 JNJ16259685
+ 3.2 JNJ16259685
%MPE
100
75
50
25
0
S
0.032
0.32
3.2
Dose Buprenorphine (mg/kg)
S
0.32
3.2
32
Dose Dezocine (mg/kg)
0 15 30 45 60 90 120
Time After Buprenorphine (min)
Time After Dezocine (min)

Figure 4.3 Buprenorphine and dezocine in combination with MPEP on thermal nociception at 56°C. Left, timecourse of 1.0 mg/kg buprenorphine alone and in combination with MPEP. Right, timecourse of 3.2 mg/kg dezocine alone and in combination with MPEP. Abscissa, time after administration of buprenorphine or dezocine. Ordinate, antinociception as percent maximum possible effect.

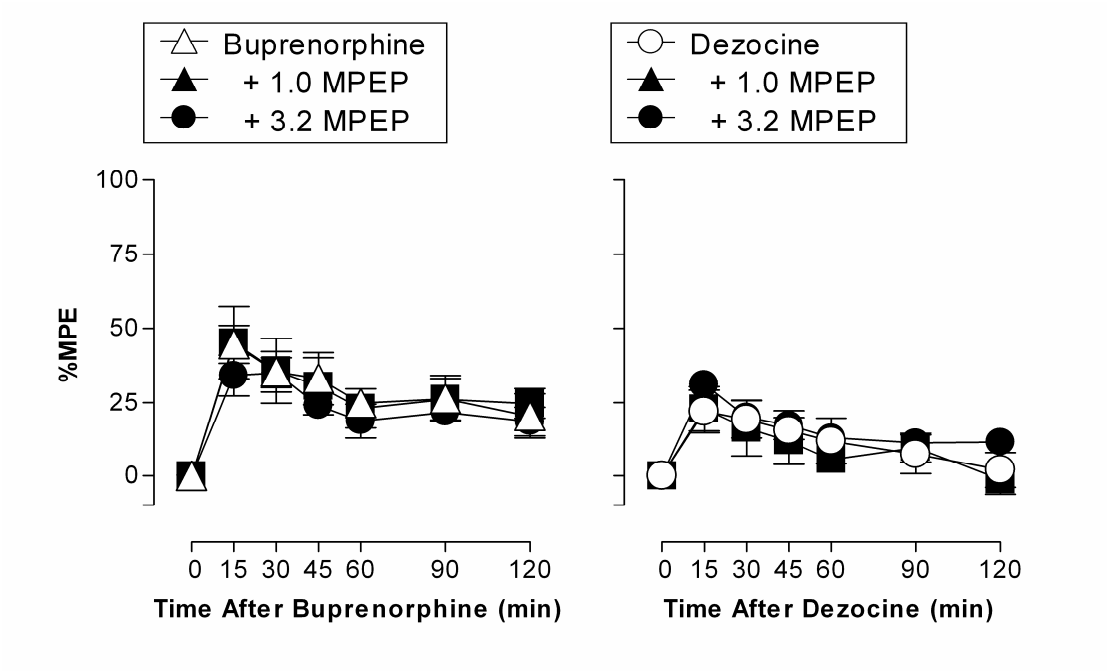


Figure 4.4 Buprenorphine and dezocine in combination with LY341495 on thermal nociception at 56°C. Top, dose effect curves for buprenorphine (left) and dezocine (right) alone and in combination with LY341495. Abscissa, dose of buprenorphine or dezocine in mg/kg. Ordinate, antinociception as percent maximum possible effect. Bottom, timecourse of 1.0 mg/kg buprenorphine (left) and 3.2 mg/kg dezocine (right) alone and in combination with LY341495. Abscissa, time after administration of buprenorphine or dezocine. Ordinate, antinociception as percent maximum possible effect.

*, Significantly different from buprenorphine or dezocine alone.

Figure 4.4

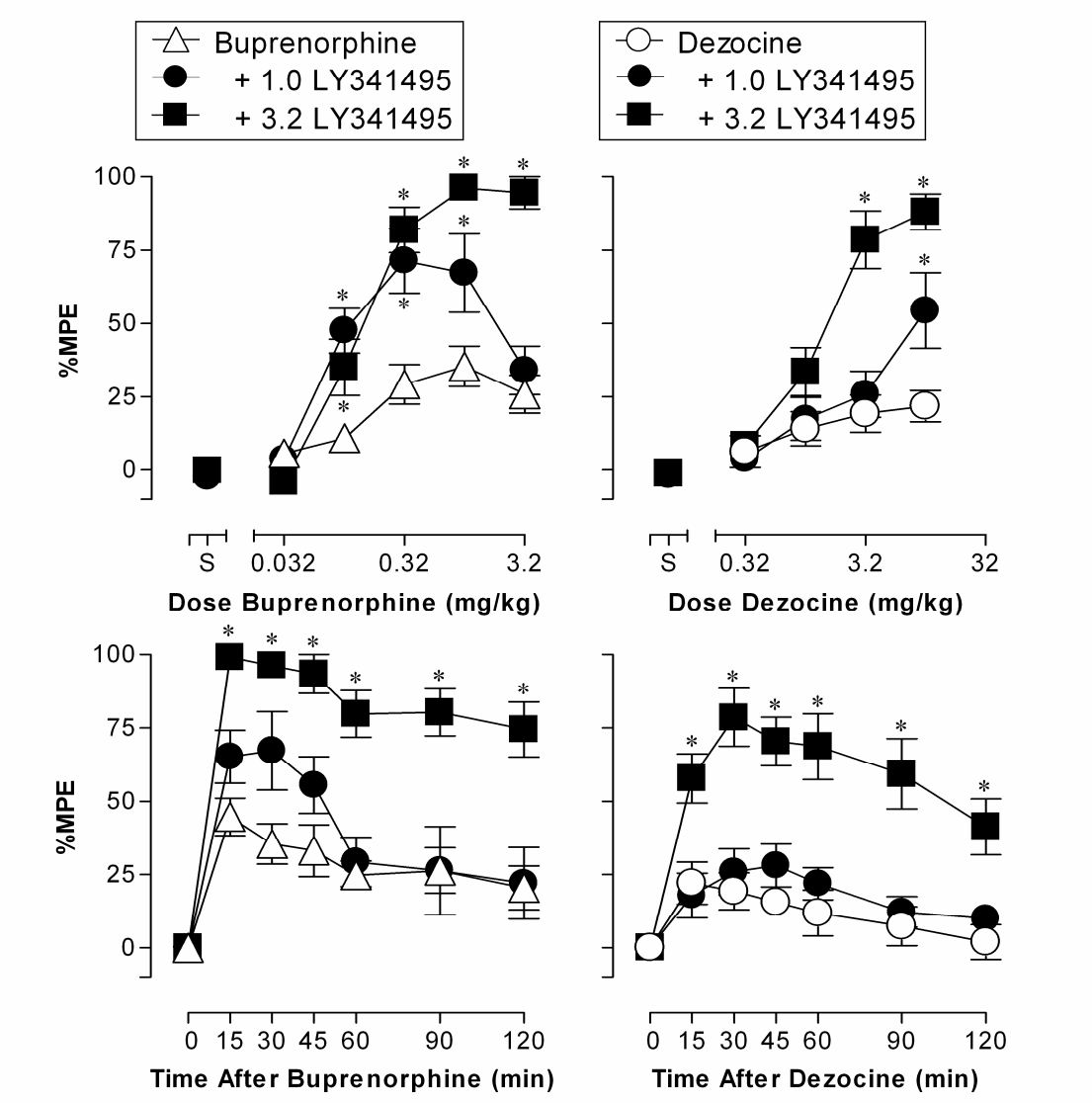
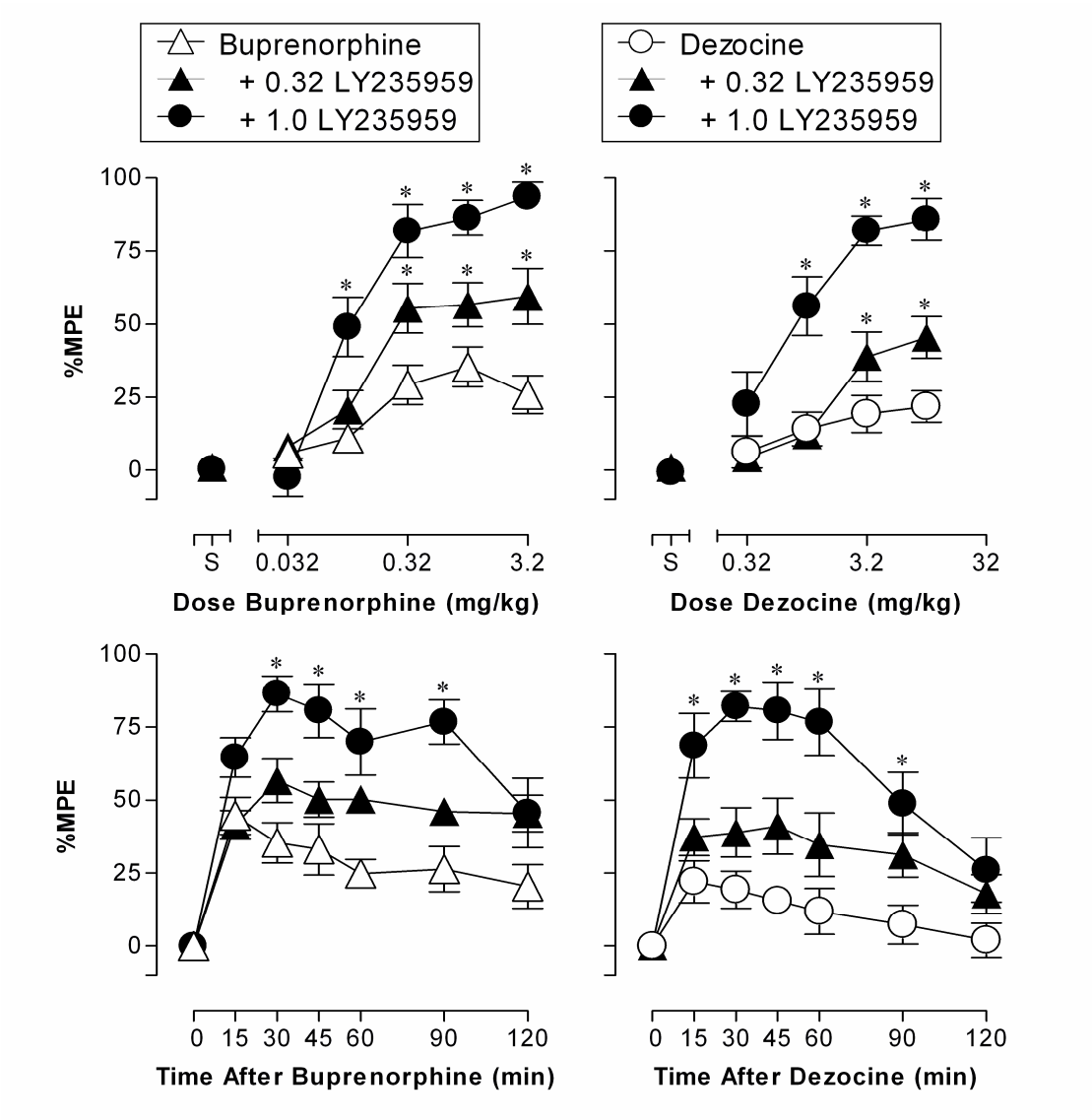

Buprenorphine
+ 1.0 LY341495
+ 3.2 LY341495
Dezocine
+ 1.0 LY341495
+ 3.2 LY341495
%MPE
100
75
50
25
0
S
0.032
0.32
3.2
Dose Buprenorphine (mg/kg)
S
0.32
3.2
32
Dose Dezocine (mg/kg)
0 15 30 45 60 90 120
Time After Buprenorphine (min)
0 15 30 45 60 90 120
Time After Dezocine (min)

Figure 4.5 Buprenorphine and dezocine in combination with LY235959 on thermal nociception at 56°C. Top, dose effect curves for buprenorphine (left) and dezocine (right) alone and in combination with LY235959. Abscissa, dose of buprenorphine or dezocine in mg/kg. Ordinate, antinociception as percent maximum possible effect. Bottom, timecourse of 1.0 mg/kg buprenorphine (left) and 3.2 mg/kg dezocine (right) alone and in combination with LY235959. Abscissa, time after administration of buprenorphine or dezocine. Ordinate, antinociception as percent maximum possible effect.

*, Significantly different from buprenorphine or dezocine alone.

Figure 4.5


Buprenorphine
+ 0.32 LY235959
+ 1.0 LY235959
Dezocine
+ 0.32 LY235959
+ 1.0 LY235959
%MPE
100
75
50
25
0
S
0.032
0.32
3.2
32
Dose Buprenorphine (mg/kg)
Dose Dezocine (mg/kg)
0 15 30 45 60 90 120
Time After Buprenorphine (min)
Time After Dezocine (min)

CHAPTER 5

GENERAL DISCUSSION

EXPERIMENTAL RESULTS

The present series of studies assessed the interactive effects of opioids and glutamate receptor antagonists in assays of schedule-controlled responding and thermal nociception. Experiment 1 examined the effects of morphine, buprenorphine, butorphanol, and nalbuphine alone and in combination with the NMDA receptor antagonist LY235959. Although previous reports have assessed the interactive effects of opioids and NMDA receptor antagonists, the purpose of this study was to analyze these interactions using a formally quantitative approach and to determine whether these interactions extend to another behavioral endpoint. The results from this study indicate that LY235959/morphine and LY235959/buprenorphine mixtures produced additive or infra-additive effects on schedule-controlled responding, whereas LY235959/butorphanol and LY235959/nalbuphine mixtures produced additive or supra-additive effects. In addition, mixtures of LY235959 in combination with morphine, buprenorphine, butorphanol, and nalbuphine produced additive or supra-additive effects on thermal nociception. Together, these data suggest that the interactive effects of opioids and NMDA receptor antagonists depend on the behavioral endpoint, the relative proportions of drugs in the mixture, and the opioid under study.

In addition to ionotropic receptors, endogenous glutamate exerts its action through a family of metabotropic glutamate (mGlu) receptors that activate various intracellular second

messenger systems through G-proteins. Experiment 2 examined the interactive effects of morphine in combination with the mGlu1 receptor antagonist JNJ16259685, the mGlu5 receptor antagonist MPEP, and the mGlu2/3 receptor antagonist LY341495 in assays of schedule-controlled responding and thermal nociception. The results from this experiment suggest that JNJ16259685, MPEP, and LY341495 produce additive effects when administered in combination with morphine on schedule-controlled responding. In contrast, JNJ16259685 and LY341495 produced supra-additive effects with morphine when assessed on thermal nociception. Together, these data implicate the mGlu receptor system in the antinociceptive effects of morphine, and suggest that interactions between mGlu receptor antagonists and morphine depend on the mGlu receptor subtype and behavioral endpoint being examined.

The purpose of Experiment 3 was to use opioid efficacy as a tool to dissociate the mechanism by which NMDA and mGlu receptor antagonists increase the antinociceptive effects of morphine and other opioids. In this experiment, morphine and the low-efficacy opioid receptor agonists buprenorphine and dezocine were first assessed in conditions of low (53°C) and high (56°C) stimulus intensity. The antinociceptive effects of buprenorphine and dezocine were dependent on the nociceptive stimulus intensity, as these opioid receptor agonists produced sub-maximal effects at 56°C. Under these conditions, buprenorphine and dezocine were assessed after pretreatment with LY235959, JNJ16259685, MPEP, and LY341495. The results from this study indicated that LY235959, JNJ16259685, and LY341495 increased the efficacy of both buprenorphine and dezocine. The findings from this experiment provide additional evidence that NMDA, mGlu1, and mGlu2/3 receptor

antagonists increase the acute antinociceptive effects of opioids, and further suggest that this enhancement results from an increase in opioid efficacy.

Taken together, these experiments assessed the interactive behavioral effects of opioid receptor agonists and glutamate receptor antagonists. The experimental results obtained from these studies suggest that these interactive effects are dependent upon factors such as the relative proportions of drugs and the experimental endpoint under study. In addition, the pharmacological affinity of the opioid and glutamate receptor antagonists being examined is an important determinant of their behavioral effects.

RELATIVE PROPORTIONS OF DRUGS AS A DETERMINANT OF DRUG INTERACTIONS

Throughout Experiments 1 and 2, drug interactions were assessed using a fixed-proportion design. This approach is advantageous for several reasons (Tallarida, 2000). First, a drug mixture manufactured for clinical use would most likely contain a fixed ratio of the drugs. In addition, in preclinical work, administration of drugs in fixed proportions simplifies the analysis of the data. Finally, previous research has demonstrated that deviation from additivity depends on the relative proportions of the drugs under study (Gessner and Cabana, 1970; Tallarida, 2000; Stevenson et al., 2003, 2005; Craft and Leitl, 2006).

In what is now a seminal study, Gessner and Cabana (1970) reported on the interactive effects of chloral hydrate and ethanol on the loss of righting reflex in mice. These authors demonstrated that supra-additive effects occurred between chloral hydrate and ethanol if the drugs were administered at a ratio equal to or greater than 1:7.2 chloral hydrate/ethanol, whereas additive effects were observed at ratios less than 1:7.2 chloral hydrate/ethanol. The results from this study were among the first to demonstrate that deviation from additivity is

not only a property of the two drugs under study, but also depends in the relative amounts of each drug in the combination tested

In the current series of experiments, drug interactions that were administered in fixed-proportions (Experiments 1 and 2) were assessed at three different drug ratios. In agreement with the findings of Gessner and Cabana (1970), the interactive effects of opioids and glutamate receptor antagonists were often dependent on the relative proportions of drugs in mixture. For example, in the assay of schedule-controlled responding, a mixture containing a ratio of 1.3:1 LY235959/morphine produced infra-additive effects, whereas a mixture containing a ratio of 12:1 LY235959/morphine produced additive effects. Similar proportion-dependent effects were observed in the assay of thermal nociception. For example, a mixture of 0.31:1 LY341495/morphine resulted in an additive interaction whereas a mixture of 0.93:1 LY341495/morphine produced supra-additive effects. Together, these data provide further evidence that the relative proportions of two drugs in a mixture is an important determinant of their behavioral effects.

BEHAVIORAL ENDPOINT AS A DETERMINANT OF DRUG INTERACTIONS

Previous research has suggested that the interactive effects of two drugs may vary as a function of the experimental endpoint under study (Gessner and Cabana, 1970; Tallarida, 2000; Stevenson et al., 2003, 2005). For example, Stevenson and colleagues reported on the interactive effects of heroin and the δ-opioid receptor agonist SNC80 in assays of thermal nociception, schedule-controlled responding, and drug self-administration (Stevenson et al., 2005). In this study, mixtures of heroin and SNC80 produced supra-additive effects on thermal nociception while producing additive effects on schedule-controlled responding.

Furthermore, these same drug mixtures produced additive effects on drug self-administration as measured under both a fixed ratio and a food versus heroin concurrent-choice schedule of reinforcement. These data demonstrate that there is a behavioral selectivity in the interactive effects of drug mixtures under certain conditions.

In Experiments 1 and 2, the interactive effects of opioid receptor agonists and mGlu receptor antagonists were assessed on two experimental endpoints: thermal nociception and schedule-controlled responding. In agreement with the aforementioned studies, the effects of mGlu receptor antagonist/opioid receptor agonist mixtures were often dependent on the behavioral endpoint under study. For example, JNJ16259685/morphine mixtures produced additive effects in the assay of schedule-controlled responding across a range of proportions. In contrast, these same drug mixtures produced supra-additive effects in the assay of thermal nociception. This finding was similar to LY235959/morphine and LY341495/morphine mixtures, and suggests that these glutamate receptor antagonists may specifically increase the antinociceptive properties of morphine relative to their rate-reducing effects.

NOCICEPTIVE ENDPOINT AS A DETERMINANT OF DRUG INTERACTIONS

In the current series of experiments, the interactive effects of opioid receptor agonists and mGlu receptor antagonists were assessed across two different measures of antinociception: a tail-flick procedure (Experiments 1 and 2) and a hot plate procedure (Experiment 3). These measures of antinociception were chosen to discriminate between spinal (tail-flick) and supraspinal (hot plate) mechanisms of the drug interactions. The tail-flick apparatus is thought to elicit nociceptive transmission directly from the tail to the dorsal horn via Aδ-fibers, resulting in one of the simple spinal reflexes through the reflex arc (Chapman et al.,

1985). The behavior required to respond on the hot plate (licking or shuffling of the hind paw) requires higher brain function, and is thought to be associated with both spinal and supraspinal involvement in nociception (Chapman et al., 1985; Dubner and Ren, 1999).

In addition to possible differences in anatomical sites, research has implicated cellular differences mediating responses between the hot plate and tail-flick procedures. Specifically, reports suggest that the serotonergic system mediates the transmission of nociceptive stimuli. Extensive studies have suggested that serotonin (5-HT) receptors are expressed in the spinal cord, and that 5-HT_{1A} and 5-HT_{1B} receptors are particularly expressed in the superficial laminae of the dorsal horn (e.g. Hamon and Bourgoin 1999). In addition, recent studies with 5-HT_{1A} and 5-HT_{1B} receptor knock-out mice suggest that these receptor subtypes may differentially mediate nociceptive transmission from the tail-flick apparatus and the hot plate (Kayser et al., 2007). Specifically, these studies suggest that 5-HT_{1A} receptors meditate behaviors on the hot plate, whereas both 5-HT_{1A} and 5-HT_{1B} receptors mediate responses in the tail-flick procedure.

The results from the present study confirm previous findings demonstrating that NMDA receptor antagonists can increase opioid-induced antinociception as measured in both hot plate (Bernardi et al., 1996; Plesan et al., 1998; Baker et al., 2002; Bulka et al., 2002) and tail-flick procedures (Mao et al., 1996; Belozertseva et al., 2000; Kozela et al., 2001; Holtman et al., 2003; Nemmani et al., 2004). In addition, the present study suggests that mGlu1 and mGlu2/3 receptor antagonists increase morphine's antinociceptive effects across both procedures. Together, these data suggest that nociceptive endpoint is not an important determinant of glutamate receptor antagonist/opioid receptor agonist interactions. Further, the results from these experiments suggest that supraspinal mechanisms are not necessary for

these interactions. Finally, the present series of studies suggest that differences in serotonergic mediation between the tail-flick and hot plate assays do not result in differential interactive effects between opioids and glutamate receptor antagonists.

OPIOID RECEPTOR AGONIST AFFINITY AS A DETERMINANT OF DRUG INTERACTIONS

The opioid receptor agonists selected for study in Experiment 1 were chosen based on their relative affinities for opioid receptor subtypes. Specifically, morphine, buprenorphine, butorphanol, and nalbuphine differ in their affinity for μ- and κ-opioid receptors. Similar to morphine, buprenorphine produces behavioral effects primarily through activation of the μ-opioid receptor (Adams et al., 1990; Walker et al., 1998, 1999). Butorphanol and nalbuphine have affinity for both μ-opioid receptors and κ-opioid receptors (Leander 1983; Gerak et al., 1994; Walker et al., 1994; Smith et al., 1999) and can act as an agonist or antagonist at these receptors depending on the experimental procedure (Leander, 1983; Dykstra 1990; Butelman et al., 1995; Vivian et al., 1999).

In the assay of schedule-controlled responding, administration of LY235959 in combination with morphine and buprenorphine resulted in an additive interaction. In contrast, some LY235959/butorphanol and LY235959/nalbuphine mixtures produced supra-additive effects. Among the low-efficacy opioids examined in Experiment 1, butorphanol and nalbuphine possess significant agonist activity at κ-opioid receptors in rodent models other than antinociception (Jaw et al., 1993; Smith and Picker, 1995; Leander et al., 1997; Craft, 2000) and in vitro (Zhu et al., 1997; Remmers et al., 1999). Therefore, it is possible that the supra-additive rate-decreasing effects are mediated, in part, by their κ component. To date,

the interactive effects of selective κ-opioid receptor agonists and NMDA antagonists have not been assessed on this endpoint.

Differences in drug interactions between the opioid agonists were also observed in the assay of thermal nociception. On this endpoint, LY235959 produced supra-additive effects with each of the opioids, regardless of pharmacological selectivity. However, although supra-additive effects of LY235959/nalbuphine mixtures with a higher proportion of LY235959 were statistically significant, the shifts in the nalbuphine dose-effect curves were modest relative to the other opioids tested. Although this difference is unclear, the degree to which nalbuphine was increased relative to the other opioids may be due to differences in pharmacological selectivity.

In antinociceptive assays, the effects of morphine, buprenorphine, butorphanol, and nalbuphine are mediated predominately through agonist activity at the μ-opioid receptor in monkeys and rodents (Zimmerman et al., 1987; Walker et al., 1993; Butelman et al., 1995; Garner et al., 1997) although nalbuphine has been shown to have some κ-opioid receptor activity in mice (Pick et al., 1992). Behavioral evidence suggests drugs with affinity for μ-opioid receptors are increased to a greater extent by NMDA antagonists relative to drugs with affinity for κ-opioid receptors (e.g. Chen et al., 2005). Therefore, although significant supra-additive effects were determined for LY235959/nalbuphine mixtures in this assay, the effects may be blunted by the differential pharmacological selectivity of nalbuphine.

GLUTAMATE RECEPTOR ANTAGONIST AFFINITY AS A DETERMINANT OF DRUG INTERACTIONS

The results from the current series of experiments suggest that the NMDA antagonist LY235959, the mGlu1 antagonist JNJ16259685, and the mGlu2/3 antagonist LY341495 increase opioid-induced antinociception. This effect was dependent on the pharmacological selectivity of the glutamate receptor antagonist under study in that the mGlu5 antagonist MPEP did not affect opioid antinociception. Together, these data suggest that glutamate receptor affinity is an important determinant of glutamate receptor antagonist/μ-opioid receptor agonist interactions.

Opioid + NMDA receptor antagonist interactions. The interactive effects of LY235959 and the opioid receptor agonists in Experiments 1 and 3 were expected, as previous research has demonstrated that NMDA receptor antagonists increase the antinociceptive effects of μ-opioid receptor agonists under numerous conditions (Mao et al., 1996; Bespalov et al., 1998; Pleasn et al., 1998; Allen and Dykstra, 2001; Bulka et al., 2002; Allen et al., 2003; Nemmani et al., 2004). From these studies and others, a cellular model for the interactive effects of NMDA receptor antagonists and μ-opioid receptor agonists has been developed. According to this model, activation of NMDA receptors contributes to the development of acute opioid tolerance through an increase in protein kinase C and subsequent reduction in the sensitivity of μ-opioid receptors (Ben-Eliyahu et al., 1992; Mao et al., 1995). This concept is based on experimental evidence showing that serum and brain concentrations of morphine last longer than morphine antinociception (Kissin et al., 1991) and is reinforced by studies demonstrating that an NMDA antagonist blocks the development of acute tolerance revealed after a second consecutive injection of an opioid (Larcher et al., 1998). Additionally, it has been demonstrated that antinociception induced by acute administration of opioids is reduced by NMDA-mediated opposing effects (Celerier et al.,

1999). The finding from the present study that the NMDA antagonist LY235959 increases the intrinsic efficacy of μ-opioid receptor agonists corroborates this model.

Opioid + mGlu1 receptor antagonist interactions. The results from Experiments 2 and 3 provide the first experimental evidence that the antinociceptive effects of opioids are increased by an mGlu1 antagonist. Although speculative, these interactive effects of JNJ16259685 and μ-opioid receptor agonists can be conceptualized through receptor-mediated processes. Both μ-opioid receptors and mGlu1 receptors are expressed postsynaptically on dorsal horn neurons in the spinal cord (Jia et al., 1999; Alvarez et al., 2000), where mGlu1 receptors are physically linked to NMDA receptors through postsynaptic density proteins (Naisbitt et al., 1999; Tu et al., 1999). Activation of mGlu1 receptors in the dorsal horn results in the potentiation of NMDA-mediated responses, through an activation of protein kinase C and a reduction of the voltage-dependent block of NMDA receptors by Mg^{2+} (Cerne and Randic, 1992; Kelso et al., 1992; Skeberdis et al., 2001). Considering the observed increases in μ-opioid receptor agonist-induced antinociception after administration of the NMDA antagonist LY235959 (results from Experiment 1), it is possible that the increases after administration of JNJ16259685 are a result of a functionally similar process.

Opioid + mGlu5 receptor antagonist interactions. Experiments 2 and 3 suggest that, in contrast to the mGlu1 receptor antagonist JNJ16259685, the mGlu5 receptor antagonist MPEP did not modulate the antinociceptive effects of morphine. As both mGlu1 and mGlu5 receptor subtypes are Group I mGlu receptors, they are both coupled to G_q protein. Activation of both mGlu1 and mGlu5 receptors results in the dissociation of the G_q protein into $G_q\alpha$ and $G_q\beta\gamma$ subunits, which elicit similar intracellular signaling processes. In view of

the functional similarities of mGlu1 and mGlu5 receptors, the additive effects of MPEP/morphine mixtures on thermal nociception are surprising. However, although the specific mechanisms enabling mGlu1 and mGlu5 receptors to differentially interact with μ-opioid receptor agonists are unclear, available data suggests at least three possibilities: 1) differences in the anatomical distribution of mGlu1 and mGlu5 receptors, 2) different organization of the receptors in cellular microdomains, and 3) different intracellular signaling regulation of the receptors by phosphorylation.

The antinociceptive effects of opioids and mGlu antagonist/opioid mixtures are thought to be mediated in the dorsal horn of the spinal cord. Although the expression of mGlu1 and mGlu5 mRNA's are abundant in the dorsal horn, localization of immunoreactivity for these mGlu receptor subtypes suggests that mGlu5 receptors may not be present in this region (Yung, 1998; but see Vidnyánszky et al., 1994). Clearly, this anatomical difference between mGlu1 and mGlu5 receptors would result in differential interactive effects with opioids in assays of nociception. Although the lack of mGlu5 immunoreactivity does not directly preclude the existence of mGlu5 receptors in the dorsal horn, this possibility illustrates how differences in the anatomical distribution of mGlu1 and mGlu5 receptors may contribute to the behavioral differences in their interaction with opioids.

In addition to differences in the distribution of mGlu1 and mGlu5 receptors at anatomical sites, these receptor subtypes may differ in their spatial distribution within a neuron. As an example, Delmas and colleagues (2002) have demonstrated a role for signaling microdomains in the induction of differential responses from receptors using similar signaling cascades. Using bradykinin B_2 and muscarinic M_1 receptors as a model, it was shown that, despite activating the same signaling pathway, activation of bradykinin receptors

results in relatively larger Ca^{2+}-mediated responses. Through a series of experiments, these authors demonstrate that the different responses mediated by the two receptor subtypes are a result of the spatial proximity of bradykinin B_2 receptors with modulatory IP_3 receptors. Together, bradykinin B_2 receptors and IP_3 receptors form signaling microdomains together, resulting in an enhanced Ca^{2+} response. In contrast, muscarinic M_1 receptors are randomly distributed, and this spatial separation causes a reduced Ca^{2+} response. Whether a related mechanism mediates the differences in mGlu1/opioid and mGlu5/opioid interactions observed in the present study remains to be determined.

In addition, the discrepancy between mGlu1 and mGlu5 receptors may be due to different regulation by phosphorylation. mGlu1 and mGlu5 receptors differ in particular sites of their amino acid sequence that account for differences in their downstream signaling processes. For example, the amino acids aspartate and threonine are located within the G-protein-interacting domains of mGlu1 and mGlu5 receptors, respectively. PKC-induced phosphorylation of the aspartate residue results in a single-peak release of Ca^{2+} from intracellular stores in cultured cells expressing the mGlu1 receptor (Kawabata et al., 1996). In cells expressing mGlu5, however, phosphorylation of the theonine residue by PKC results in an oscillatory release of Ca^{2+} (Kawabata et al., 1996; Nakanishi et al., 1998; Nash et al., 2002). Although the implications of these or other differences in phosphorylation on mGlu5/opioid receptor agonist interactions are not known, differences in the specific biochemical mechanisms underlying the responses of mGlu1 and mGlu5 receptors may contribute to their different interactive effects with opioids.

It is also important to note that the lack of an effect after administration of MPEP may be due to the specific experimental conditions under which MPEP/opioid interactions were

assessed. As mentioned above, the behavioral effects of two drugs may depend on the experimental endpoint under study and on the proportion of drugs in the mixture (Gessner and Cabana, 1970; Tallarida, 2000; Stevenson et al., 2003, 2005; Craft and Leitl, 2006). Indeed, a growing body of experimental evidence indicates that MPEP modulates the effects of opioids on other endpoints (Popik and Wrobel, 2002; Kozela et al., 2003; Smith et al., 2004), and different results may be obtained if assessed in other nociceptive assays. Although the current set of experiments failed to show an effect in both a hot plate and tail-flick procedure, this does not rule out the possibility that MPEP/opioid mixtures may interact in other assays of nociception. Additional studies assessing the interactive effects of MPEP and opioids in other proportions or in other assays of thermal nociception could yield results that are different from those reported here.

Regardless of the mechanism underlying the differences in mGlu1/opioid and mGlu5/opioid interactions, these data suggest that despite the functional similarities of mGlu1 and mGlu5 receptors, the manner in which they interact with morphine on thermal nociception may be fundamentally different.

Opioid + mGlu2/3 receptor antagonist interactions. In addition to the results obtained with the MPEP/morphine interactions, the supra-additive interaction between LY341495 and the opioid receptor agonists observed in Experiments 2 and 3 were unexpected (but see Yoon et al., 2006). Numerous reports have demonstrated that mGlu2 and mGlu3 receptors are located on presynaptic neurons in numerous brain regions, and drugs that act as *agonists* at these receptors decrease glutamate release and subsequent postsynaptic binding to both iGlu and mGlu receptor subtypes (Pin and Duvoisin, 1995). In agreement with these reports, some behavioral data suggest that mGlu2/3 agonists inhibit the development of tolerance and

dependence in a similar manner as NMDA antagonists (Klodzinska et al., 1999; Popik et al., 2000).

In the dorsal horn, both pre- and post-synaptic mGlu2 and mGlu3 receptors have been identified and, as a result, conflicting results are often reported from both *in vitro* and *in vivo* preparations (Carlton et al., 2001), For example, mGlu2/3 agonists produce both facilitation and inhibition of neuronal activity (Bond and Lodge, 1995; Cao et al., 1995; King and Liu, 1996), and both agonism and antagonism of mGlu2/3 receptors decrease pain related behaviors in animal models of inflammation (Jones et al., 2005; Yoon et al., 2006). These results may reflect the complex interaction of pre- and post-synaptic mGlu2 and mGlu3 receptors in the dorsal horn, and make the interpretation of mGlu2/3-mediated increases in opioid efficacy observed in the current study difficult.

The understanding of mGlu2/3 antagonist/opioid interactions is further limited by the selectivity of available mGlu2/3 antagonists. In contrast to available noncompetitive antagonists of group I mGlu receptors, only competitive antagonists have been developed for group II mGlu receptors. As a result of the high level of conservation of the orthosteric binding site between mGlu receptor subtypes, the selectivity of these competitive mGlu2/3 antagonists is compromised (Pin and Duvoisin, 1995). LY341495 is among the most selective mGlu2/3 antagonists reported to date, with optimal affinity for mGlu2 and mGlu3 receptors relative to compounds with structural similarity (Ornstein et al., 1998). LY341495 possesses antagonist selectivity for mGlu receptors with a potency order of mGlu3 >= mGlu2 > mGlu8 > mGlu7 >> mGlu1 = mGlu5 > mGlu4, with inhibitory activity at mGlu1 and mGlu5 receptors at 50-500 fold higher doses than mGlu2 and mGlu3 receptors (Kingston et al., 1998). However, it is worthy to note that at greater than micromolar concentrations,

LY341495 and other available mGlu2/3 antagonists may show antagonist activity across other mGlu receptors. As more selective mGlu2/3 antagonists become available (e.g. Hemstapat et al., 2007), further research is necessary to elucidate the mechanisms mediating mGlu2/3 antagonist/morphine interactions.

IMPLICATIONS

The primary intention of assessing the behavioral effects of opioids alone and in combination with glutamate receptor antagonists in the present series of experiments was to enhance the understanding of the pharmacological nature of glutamate/opioid interactions. However, the findings from these experiments may also have important clinical implications. Together, the experiments described here may provide a basis for the assessment of glutamate receptor antagonist/opioid receptor agonist combinations in animal models of more clinically relevant pain states (e.g. models of inflammation, hyperalgesia, allodynia, etc.) and ultimately for the assessment of these combinations in a human population.

Throughout Experiments 1 and 2, the interactive effects of opioid agonists and glutamate receptor antagonists were assessed in assays of thermal nociception and schedule controlled responding. These two endpoints were chosen to discriminate the interactive effects of glutamate antagonist/opioid mixtures on the targeted behavior of antinociception, rather than a general modulation of all behavioral effects. The results from these studies demonstrate that studying drug interactions across a range of relative proportions and across experimental endpoints can reveal characteristics of these interactions that may have clinical potential. Specifically, these data suggest that it is possible to develop glutamate antagonist/opioid

mixtures that interact in a supra-additive manner specifically at brain regions that mediate the targeted behavior of antinociception.

Clinical interest in combining NMDA and mGlu receptor antagonists with opioids that are lower in efficacy for the treatment of pain may also arise from the results of Experiment 1 and 3. Although low-efficacy opioid agonists are less effective than morphine (e.g. Dykstra, 1990; Walker et al., 1993; Morgan et al., 1999), they are often characterized as having a lower potential for abuse and an improved side-effect profile (Hoskin and Hanks, 1991; Preston and Jasinski, 1991). The results from Experiment 1 suggest that an NMDA antagonist and low-efficacy opioids produce supra-additive effects on thermal nociception. In addition, the results from Experiment 3 suggest that NMDA and mGlu receptor antagonists increase the efficacy of low-efficacy opioids when assessed at high stimulus intensities. Together, these results demonstrate that glutamate receptor antagonists can increase the antinociceptive effects of low-efficacy opioids, suggesting that these drug combinations may be useful for various pain states.

Although mGlu receptor antagonist/opioid combinations have not been assessed clinically, NMDA receptor antagonists are well tolerated in human populations when administered in combination with opioid agonists for the treatment of pain (Goldblum, 2000). To date, a majority of the efforts to develop combination medications have focused on the administration of the noncompetitive NMDA receptor antagonists dextromethorphan and ketamine in conjunction with morphine and fentanyl treatment (Monck, 2003; Tucker et al., 2005). The results from the current series of experiments suggest that a competitive NMDA receptor antagonist can increase the antinociceptive effects of other opioids, regardless of efficacy at the μ-opioid receptor. In addition, these experiments suggest that mGlu receptor

antagonists can also increase opioid antinociception, providing alternative glutamate sites as modulators of opioid antinociception. Together, these data suggest that combining NMDA or mGlu receptor antagonists with μ-opioid receptor agonists may prove useful for the treatment of pain.

REFERENCES

Adams JU, Paronis CA, and Holtzman SG (1990) Assessment of relative intrinsic activity of mu-opioid analgesics in vivo by using beta-funaltrexamine. *J Pharmacol Exp Ther* **255:**1027-1032.

Alvarez FJ, Villalba RM, Carr PA, Grandes P and Somohano PM (2000) Differential distribution of metabotropic glutamate receptors 1a, 1b, and 5 in the rat spinal cord. *J Comp Neurol* **422:**464-487.

Allen RM, Dykstra LA (2001) *N*-methyl-D-aspartate receptor antagonists potentiate the antinociceptive effects of morphine in squirrel monkeys. *J Pharmacol Exp Ther* **298:**288-297.

Allen RM, Granger AL, and Dykstra LA (2002) Dextromethorphan potentiates the antinociceptive effects of morphine and the δ-opioid agonist SNC80 in squirrel monkeys. *J Pharmacol Exp Ther* **300:**435-441.

Allen RM, Granger AL, and Dykstra LA (2003) The competitive *N*-Methyl-D-aspartate receptor antagonist (-)-6-phosphonomethyl-deca-hydroisoquinoline-3-carboxylic acid (LY235959) potentiates the antinociceptive effects of opioids that vary in efficacy at the μ-opioid receptor. *J Pharmacol Exp Ther* **307:**785-792.

Aoki T, Narita M, Shibasaki M, and Suzuki T (2004) Metabotropic glutamate receptor 5 localized in the limbic forebrain is critical for the development of morphine-induced rewarding effect in mice. *Eur J Neurosci* **20:**1633-1638.

Baker AK, Hoffmann VL, and Meert TF (2002) Dextromethorphan and ketamine potentiate the antinociceptive effects of mu- but not delta- or kappa-opioid agonists in a mouse model of acute pain. *Pharmacol Biochem Behav* **74:**73-86.

Belozertseva IV, Dravolina OA, Neznanova ON, Danysz W, and Bespalov AY (2000) Antinociceptive activity of combination of morphine and NMDA receptor antagonists depends on the inter-injection interval. *Eur J Pharmacol* **396:**77-83.

Ben-Eliyahu S, Marek P, Vaccarino AL, Mogil JS, Sternberg WF, and Liebeskind JC (1992) The NMDA receptor antagonist MK-801 prevents long-lasting non-associative morphine tolerance in the rat. *Brain Res* **575:**304-308.

Bernardi M, Bertolini A, Szczawinska K, and Genedani S (1996) Blockade of the polyamine site of NMDA receptors produces antinociception and enhances the effect of morphine, in mice. *Eur J Pharmacol* **298:**51-55.

Bliss CI (1939) The toxicity of poisons applied jointly. *Ann Appl Biol* **26:**585-615.

Bond A and Lodge D (1995) Pharmacology of metabotropic glutamate receptor-mediated enhancement of responses to excitatory and inhibitory amino acids on rat spinal neurones in vivo. *Neuropharmacology* **34:**1015-1023.

Bossard AE, Guirimand F, Fletcher D, Gaude-Joindreau V, Chauvin M, and Bouhassira D (2002) Interaction of a combination of morphine and ketamine on the nociceptive flexion reflex in human volunteers. *Pain* **98:**47-57.

Bulka A, Wiesenfeld-Hallin Z, and Xu XJ (2002) Differential antinociception by morphine and methadone in two sub-strains of Sprague-Dawley rats and its potentiation by dextromethorphan. *Brain Res* **942:**95-100.

Butelman ER, Winger G, Zernig G, and Woods JH (1995) Butorphanol: characterization of agonist and antagonist effects in rhesus monkeys. *J Pharmacol Exp Ther* **272:**845-853.

Cao CQ, Evans RH, Headley PM, and Udvarhelyi PM (1995) A comparison of the effects of selective metabotropic glutamate receptor agonists on synaptically evoked whole cell currents of rat spinal ventral horn neurones in vitro. *Br J Pharmacol* **115:**1469-1474.

Carlton SM, Hargett GL, and Coggeshall RE (2001) Localization of metabotropic glutamate receptors 2/3 on primary afferent axons in the rat. *Neuroscience* **105:**957-969.

Carroll FY, Stolle A, Beart PM, Voerste A, Brabet I, Mauler F, Joly C, Antonicek H, Bockaert J, Müller T, Pin JP, and Prézeau L (2001) BAY36-7620: a potent non-competitive mGlu1 receptor antagonist with inverse agonist activity. *Mol Pharmacol* **59:**965-973.

Carter AJ (1994) Many agents that antagonize the NMDA receptor-channel complex *in vivo* also cause disturbances of motor coordination. *J Pharmacol Exp Ther* **269:**573-580.

Celerier E, Laulin J, Larcher A, Le Moal M, and Simonnet G (1999) Evidence for opiate-activated NMDA processes masking opiate analgesia in rats. *Brain Res* **847:**18-25.

Cerne R and Randic M (1992) Modulation of AMPA and NMDA responses in rat spinal dorsal horn neurons by trans-1-aminocyclopentane-1,3-dicarboxylic acid. *Neurosci Lett* **144:**180-184.

Chapman CR, Casey KL, Dubner R, Foley KM, Gracely RH, and Reading AE (1985) Pain measurement: An overview. *Pain* **22:**1–31.

Chen SL, Huang EY, Chow LH, Tao PL (2005) Dextromethorphan differentially affects opioid antinociception in rats. *Br J Pharmacol* **144:**400-404.

Chen JC, Smith ER, Cahill M, Cohen R, and Fishman JB (1992) The opioid receptor binding of dezocine, morphine, fentanyl, butorphanol and nalbuphine. *Life Sci* **52:**389–396.

Craft RM and Lee DA (2005) NMDA antagonist modulation of morphine antinociception in female vs. male rats. *Pharmacol Biochem Behav* **80:**639-649.

Craft RM and Leitl MD (2006) Potentiation of morphine antinociception by pentobarbital in female vs. male rats. *Pain* **121:**115-125.

Craft RM, Ulibarri CM, and Raub DJ (2000) Kappa opioid-induced diuresis in female vs. male rats. *Pharmacol Biochem Behav* **65:**53–59

Delmas P, Wanaverbecq N, Abogadie FC, Mistry M, and Brown DA (2002) Signaling microdomains define the specificity of receptor-mediated InsP(3) pathways in neurons. *Neuron* **34:**209-220.

Dubner R and Ren K (1999) Assessing transient and persistent pain in animals, in *Textbook of Pain* (Wall PD and Melzack R eds) pp 359-369, Churchill Livingstone, Edinburgh, UK.

Dykstra LA (1990) Butorphanol, levallorphan, nalbuphine and nalorphine as antagonists in the squirrel monkey. *J Pharmacol Exp Ther* **254:**245-252.

Emmerson PJ, Clark MJ, Mansour A, Akil H, Woods JH, and Medzihradsky F (1996) Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the μ opioid receptor. *J Pharmacol Exp Ther* **278:**1121-1127.

Farlow MR (2004) NMDA receptor antagonists: A new therapeutic approach for Alzheimers disease. *Geriatrics* **59**:22–27.

Finney DJ (1942) The analysis of toxicity tests on mixtures of poisons. *Ann Appl Biol* **29:**82-94.

Fischer BD, Carrigan KA, and Dykstra LA (2005) Effects of *N*-methyl-D-aspartate receptor antagonists on acute morphine-induced and l-methadone-induced antinociception in mice. *J Pain* **6:**425-433.

Foltin RW, Woolverton WL, and Schuster CR (1983) Effects of d-amphetamine and haloperidol alone and in combination on milk drinking in rats. *Psychopharmacology* **80:**342-344.

Fundytus ME, Yashpal K, Chabot JG, Osborne MG, Lefebvre CD, Dray A, Henry JL, and Coderre TJ (2001) Knockdown of spinal metabotropic glutamate receptor 1 (mGluR(1)) alleviates pain and restores opioid efficacy after nerve injury in rats. *Br J Pharmacol* **132:**354-367.

Galer BS, Lee D, Ma T, Nagle B, and Schlagheck TG (2005) MorphiDex (morphine sulfate/dextromethorphan hydrobromide combination) in the treatment of chronic pain: three multicenter, randomized, double-blind, controlled clinical trials fail to demonstrate enhanced opioid analgesia or reduction in tolerance. *Pain* **115:**284-295.

Garner HR, Burke TF, Lawhorn CD, Stoner JM, and Wessinger WD (1997) Butorphanol-mediated antinociception in mice: partial agonist effects and mu receptor involvement. *J Pharmacol Exp Ther* **282:**1253-1261.

Gasparini F, Lingenhohl K, Stoehr N, Flor PJ, Heinrich M, Vranesic I, Biollaz M, Allgeier H, Heckendorn R, Urwyler S, Varney MA, Johnson EC, Hess SD, Rao SP, Sacaan AI, Santori EM, Velicelebi G, and Kuhn R (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology* **38:**1493–1503.

Gerak LR, Butelman ER, Woods JH, and France CP (1994) Antinociceptive and respiratory effects of nalbuphine in rhesus monkeys. *J Pharmacol Exp Ther* **271:**993-999.

Gessner PK and Cabana BE (1970) A study of the interaction of the hypnotic effects and of the toxic effects of chloral hydrate and ethanol. *J Pharmacol Exp Ther* **174:**247-259.

Geter-Douglass B and Witkin JM (1999) Behavioral effects and anticonvulsant efficacies of low-affinity, uncompetitive NMDA antagonists in mice. *Psychopharmacology* **146:**280-289.

Gharagozlou P, Demirci H, David Clark J, Lameh J (2003) Activity of opioid ligands in cells expressing cloned mu opioid receptors. *BMC Pharmacol* **3:**1.

Grabovsky Y and Tallarida RJ (2004) Isobolographic analysis for combinations of a full and partial agonist: curved isoboles. *J Pharmacol Exp Ther* **310:**981-986.

Hamon M and Bourgoin S (1999) Serotonin and its receptors in pain control, in *Novel Aspects of Pain Management: Opioids and Beyond* (Sawynok J and Cowan A eds) pp 203–228, John Wiley & sons Inc., New York.

Hemstapat K, Da Costa H, Nong Y, Brady AE, Luo Q, Niswender CM, Tamagnan GD, and Conn PJ (2007) A novel family of potent negative allosteric modulators of group II metabotropic glutamate receptors. *J Pharmacol Exp Ther* **322:**254-64.

Holtman JR, Jr., Jing X, and Wala EP (2003) Sex-related differences in the enhancement of morphine antinociception by NMDA receptor antagonists in rats. *Pharmacol Biochem Behav* **76:**285-293.

Hoskins PJ and Hanks GW (1991) Opioid agonist-antagonist drugs in acute and chronic pain states. *Drugs* **41:**326-344.

Javery KB, Ussery TW, Steger HG, and Colclough GW (1996) Comparison of morphine and morphine with ketamine for postoperative analgesia. *Can J Anaesth* **43:**212-215.

Jaw SP, Makimura M, Hoskins B, and Ho IK (1993) Effects of nor-binaltorphimine on butorphanol dependence. *Eur J Pharmacol* **239:**133-140.

Jeziorski M, White FJ, and Wolf ME (1994) MK-801 prevents the development of behavioral sensitization during repeated morphine administration. *Synapse* **16:**137-147.

Jia H, Rustioni A, and Valtschanoff JG (1999) Metabotropic glutamate receptors in superficial laminae of the rat dorsal horn. *J Comp Neurol* **410:**627-642.

Jones CK, Eberle EL, Peters SC, Monn JA, and Shannon HE (2005) Analgesic effects of the selective group II (mGlu2/3) metabotropic glutamate receptor agonists LY379268 and LY389795 in persistent and inflammatory pain models after acute and repeated dosing. *Neuropharmacology* **49:**206-218.

Kawabata S, Tsutsumi R, Kohara A, Yamaguchi T, Nakanishi S, and Okada M (1996) Control of calcium oscillations by phosphorylation of metabotropic glutamate receptors. *Nature* **383:**89-92.

Kayser V, Elfassi IE, Aubel B, Melfort M, Julius D, Gingrich JA, Hamon M, and Bourgoin S (2007) Mechanical, thermal and formalin-induced nociception is differentially altered in 5-HT1A-/-, 5-HT1B-/-, 5-HT2A-/-, 5-HT3A-/- and 5-HTT-/- knock-out male mice. *Pain* **130:**235-48.

Kelso SR, Nelson TE, and Leonard JP (1992) Protein kinase C-mediated enhancement of NMDA currents by metabotropic glutamate receptors in Xenopus oocytes. *J Physiol* **449:**705-718.

Kew JN and Kemp JA (1998) An allosteric interaction between the NMDA receptor polyamine and ifenprodil sites in rat cultured cortical neurones. *J Physiol* **512:**17–28.

Kew JN and Kemp JA (2005) Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology* **179:**4-29.

King AE and Liu XH (1996) Dual action of metabotropic glutamate receptor agonists on neuronal excitability and synaptic transmission in spinal ventral horn neurons in vitro. *Neuropharmacology* **35:**1673-1680.

Kingston AE, Ornstein PL, Wright RA, Johnson BG, Mayne NG, Burnett JP, Belagaje R, Wu S, and Schoepp DD (1998) LY341495 is a nanomolar potent and selective antagonist of group II metabotropic glutamate receptors. *Neuropharmacology* **37:**1-12.

Kissin I, Brown PT, Robinson A, and Bradley EL (1991) Acute tolerance in morphine analgesia: continuous infusion and single injection in rats. *Anesthesiology* **74:**166-171.

Klodzinska A, Chojnacka-Wojcik E, Palucha A, Branski P, Popik P, and Pilc A (1999) Potential anti-anxiety, anti-addictive effects of LY 354740, a selective group II glutamate metabotropic receptors agonist in animal models. *Neuropharmacology* **38:**1831-1839.

Koek W and Colpaert FC (1990) Selective blockade of N-methyl-D-aspartate (NMDA)-induced convulsions by NMDA antagonists and putative glycine antagonists: relationship with phencyclidine-like behavioral effects. *J Pharmacol Exp Ther* **252:**349-357.

Kozela E, Danysz W, and Popik P (2001) Uncompetitive NMDA receptor antagonists potentiate morphine antinociception recorded from the tail but not from the hind paw in rats. *Eur J Pharmacol* **423:**17-26.

Kozela E, Pilc A, and Popik P (2003) Inhibitory effects of MPEP, an mGluR5 antagonist, and memantine, an *N*-methyl-D-aspartate receptor antagonist, on morphine antinociceptive tolerance in mice. *Psychopharmacology* **165:**245-251.

Larcher A, Laulin JP, Celerier E, Le MM, and Simonnet G (1998) Acute tolerance associated with a single opiate administration: involvement of N-methyl-D-aspartate-dependent pain facilitatory systems. *Neuroscience* **84:**583-589.

Lavreysen H, Wouters R, Bischoff F, Nobrega Pereira S, Langlois X, Blokland S, Somers M, Dillen L, and Lesage AS (2004) JNJ16259685, a highly potent, selective and systemically active mGlu1 receptor antagonist. *Neuropharmacology* **47:**961-972.

Leander JD (1983) Evidence that nalorphine, butorphanol and oxilorphan are partial agonists at a κ-opioid receptor. *Eur J Pharmacol* **86:**467–470.

Leander JD, Hart JC, and Zerbe RL (1987) Kappa agonist-induced diuresis: evidence for stereoselectivity, strain differences, independence of hydration variables and a result of decreased plasma vasopressin levels. *J Pharmacol Exp Ther* **242:**33–39.

Loewe S (1953) The problem of synergism and antagonism of combined drugs. *Arzneimittelforsch* **3:**285-290.

Mao J, Price DD, Caruso FS, and Mayer DJ (1996) Oral administration of dextromethorphan prevents the development of morphine tolerance and dependence in rats. *Pain* **67:**361-368.

Mao J, Price DD, and Mayer DJ (1995) Mechanisms of hyperalgesia and morphine tolerance: a current view of their possible interactions. *Pain* **62:**259-274.

Monck N (2003) Morphine/dextromethorphan-endo: E 3231, Morphidex. *Drugs R D* **4:**55-56.

Morgan D, Cook CD, Smith MA, and Picker MJ (1999) An examination of the interactions between the antinociceptive effects of morphine and various μ-opioids: the role of intrinsic efficacy and stimulus intensity. *Anesth Analg* **88:**407-413.

Naisbitt S, Kim E, Tu JC, Xiao B, Sala C, Valtschanoff J, Weinberg RJ, Worley PF, and Sheng M (1999) Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. *Neuron* **23:**569-582.

Nakanishi S, Nakajima Y, Masu M, Ueda Y, Nakahara K, Watanabe D, Yamaguchi S, Kawabata S, and Okada M (1998) Glutamate receptors: brain function and signal transduction. *Brain Res Brain Res Rev* **26:**230-235.

Nash MS, Schell MJ, Atkinson PJ, Johnston NR, Nahorski SR, Challiss RA (2002) Determinants of metabotropic glutamate receptor-5-mediated Ca2+ and inositol 1,4,5-trisphosphate oscillation frequency. Receptor density versus agonist concentration. *J Biol Chem* **277:**35947-35960.

Nemmani KV, Grisel JE, Stowe JR, Smith-Carliss R, and Mogil JS (2004) Modulation of morphine analgesia by site-specific *N*-methyl-D-aspartate receptor antagonists: dependence on sex, site of antagonism, morphine dose, and time. *Pain* **109:**274-283.

Neugebauer V (2002) Metabotropic glutamate receptors--important modulators of nociception and pain behavior. *Pain* **98:**1-8.

Ornstein PL, Bleisch TJ, Arnold MB, Kennedy JH, Wright RA, Johnson BG, Tizzano JP, Helton DR, Kallman MJ, Schoepp DD, and Hérin M (2008) 2-substituted (2SR)-2-amino-2-((1SR,2SR)-2-carboxycycloprop-1-yl)glycines as potent and selective antagonists of group II metabotropic glutamate receptors. 2. Effects of aromatic substitution, pharmacological characterization, and bioavailability. *J Med Chem* **41:**358-378.

Papp M, Gruca P, and Willner P (2002) Selective blockade of drug-induced place preference conditioning by ACPC, a functional NDMA-receptor antagonist. *Neuropsychopharmacology* **27:**727-743.

Pick CG, Paul D, and Pasternak GW (1992) Nalbuphine, a mixed kappa 1 and kappa 3 analgesic in mice. *J Pharmacol Exp Ther* **262:**1044-1050.

Pin JP and Duvoisin R (1995) The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* **34:**1-26.

Pitts RC, West JP, Morgan D, Dykstra LA, and Picker MJ (1996) Opioids and rate of positively reinforced behavior: differential antagonism by naltrexone. *Behav Pharmacol* **7:**205-215.

Plesan A, Hedman U, Xu XJ, and Wiesenfeld-Hallin Z (1998) Comparison of ketamine and dextromethorphan in potentiating the antinociceptive effect of morphine in rats. *Anesth Analg* **86:**825-829.

Popik P, Kozela E, and Pilc A (2000) Selective agonist of group II glutamate metabotropic receptors, LY354740, inhibits tolerance to analgesic effects of morphine in mice. *Br J Pharmacol* **130:**1425-1431.

Popik P and Wrobel M (2002) Morphine conditioned reward is inhibited by MPEP, the mGluR5 antagonist. *Neuropharmacology* **43:**1210-1217.

Preston KL and Jasinski DR (1991) Abuse liability studies of opioid agonist-antagonists in humans. *Drug Alcohol Depend* **28:**49-82.

Rasmussen K, Martin H, Berger JE, and Seager MA (2005) The mGlu5 receptor antagonists MPEP and MTEP attenuate behavioral signs of morphine withdrawal and morphine-withdrawal-induced activation of locus coeruleus neurons in rats. *Neuropharmacology* **48:**173-180.

Remmers AE, Clark MJ, Mansour A, Akil H, Woods JH, and Medzihradsky F (1999) Opioid efficacy in a C6 glioma cell line stably expressing the human kappa opioid receptor. *J Pharmacol Exp Ther* **288:**827–833

Schoepp DD, Ornstein PL, Salhoff CR, and Leander JD (1991) Neuroprotectant effects of LY274614, a structurally novel systemically active competitive NMDA receptor antagonist. *J Neural Transm* **85:**131-143.

Skeberdis VA, Lan J, Opitz T, Zheng X, Bennett MV, Zukin RS (2001) mGluR1-mediated potentiation of NMDA receptors involves a rise in intracellular calcium and activation of protein kinase C. *Neuropharmacology* **40:**856-865.

Sladeczek F, Pin JP, Recasens M, Bockaert J, and Weiss S (1985) Glutamate stimulates inositol phosphate formation in striatal neurones. *Nature* **317:**717–719.

Smith MA and Picker MJ (1995) Examination of the kappa agonist and antagonist properties of opioids in the rat drug discrimination procedure: influence of training dose and intrinsic efficacy. *Behav Pharmacol* **6:**703–717.

Smith MA, Barrett AC, amd Picker MJ (1999) Antinociceptive effects of opioids following acute and chronic administration of butorphanol: influence of stimulus intensity and relative efficacy at the mu receptor. *Psychopharmacology* **143:**261-269.

Smith FL, Smith PA, Dewey WL, and Javed RR (2004) Effects of mGlu1 and mGlu5 metabotropic glutamate antagonists to reverse morphine tolerance in mice. *Eur J Pharmacol* **492:**137-142.

Stevenson GW, Folk JE, Linsenmayer DC, Rice KC, and Negus SS (2003) Opioid interactions in rhesus monkeys: effects of δ + μ and δ + κ agonists on schedule-controlled responding and thermal nociception. *J Pharmacol Exp Ther* **307:**1054-1064.

Stevenson GW, Folk JE, Rice KC, and Negus SS (2005) Interactions between δ and μ Opioid Agonists in Assays of Schedule-Controlled Responding, Thermal Nociception, Drug Self-Administration, and Drug versus Food Choice in Rhesus Monkeys: Studies with SNC80 [(+)-

4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide] and Heroin. *J Pharmacol Exp Ther* **314:**221-231.

Suzuki M, Kinoshita T, Kikutani T, Yokoyama K, Inagi T, Sugimoto K, Haraguchi S, Hisayoshi T, and Shimada Y (2005) Determining the plasma concentration of ketamine that enhances epidural bupivacaine-and-morphine-induced analgesia. *Anesth Analg* **101:**777-784.

Tallarida RJ (2000) *Drug Synergism and Dose-Effect Data Analysis*, Chapman & Hall/CRC Press, Boca Raton, FL.

Toll L (1995) Intact cell binding and the relation to opioid activities in SH-SY5Y cells. *J Pharmacol Exp Ther* 2**73:**721-727.

Trujillo KA and Akil H (1991) Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* **251:**85-87.

Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M, and Worley PF (1999) Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron* **23:**583-592.

Tucker AP, Kim YI, Nadeson R, and Goodchild CS (2005) Investigation of the potentiation of the analgesic effects of fentanyl by ketamine in humans: a double-blinded, randomised, placebo controlled, crossover study of experimental pain. *BMC Anesthesiol* **5:**2.

Varty GB, Grilli M, Forlani A, Fredduzzi S, Grzelak ME, Guthrie DH, Hodgson RA, Lu SX, Nicolussi E, Pond AJ, Parker EM, Hunter JC, Higgins GA, Reggiani A, and Bertorelli R (2005) The antinociceptive and anxiolytic-like effects of the metabotropic glutamate receptor 5 (mGluR5) antagonists, MPEP and MTEP, and the mGluR1 antagonist, LY456236, in rodents: a comparison of efficacy and side-effect profiles. *Psychopharmacology* **179:**207-217.

Vidnyanszky Z, Hamori J, Negyessy L, Rüegg D, Knöpfel T, Kuhn R, and Gorcs TJ (1994) Cellular and subcellular localization of the mGluR5a metabotropic glutamate receptor in rat spinal cord. *NeuroReport* **6:**209-213

Vivian JA, DeYoung MB, Sumpter TL, Traynor JR, Lewis JW and Woods JH (1999) κ-opioid receptor effects of butorphanol in rhesus monkeys. *J Pharmacol Exp Ther* **290:**259–265.

Walker K, Bowes M, Panesar M, Davis A, Gentry C, Kesingland A, Gasparini F, Spooren W, Stoehr N, Pagano A, Flor PJ, Vranesic I, Lingenhoehl K, Johnson EC, Varney M, Urban L, and Kuhn R (2001) Metabotropic glutamate receptor subtype 5 (mGlu5) and nociceptive function. I. Selective blockade of mGlu5 receptors in models of acute, persistent and chronic pain. *Neuropharmacology* **40:**1-9.

Walker EA, Butelman ER, DeCosta BR, and Woods JH (1993) Opioid thermal antinociception in rhesus monkeys: receptor mechanisms and temperature dependency. *J Pharmacol Exp Ther* **267:**280-286.

Walker EA, Makhay MM, House JD, and Young AM (1994) In vivo apparent pA2 analysis for naltrexone antagonism of discriminative stimulus and analgesic effects of opiate agonists in rats. *J Pharmacol Exp Ther* **271:**959-968.

Walker EA, Zernig G, and Young AM (1998) In vivo apparent affinity and efficacy estimates for mu opiates in a rat tail-withdrawal assay. *Psychopharmacology* **136:**15-23.

Walker EA, Tiano MJ, Benyas SI, Dykstra LA, and Picker MJ (1999). Naltrexone and beta-funaltrexamine antagonism of the antinociceptive and response rate-decreasing effects of morphine, dezocine, and d-propoxyphene. *Psychopharmacology* **144:**45-53.

Wessinger WD (1986) Approaches to the study of drug interactions in behavioral pharmacology. *Neurosci Biobehav Rev* **10:**103–113.

Woolverton WL (1987) Analysis of drug interactions in behavioral pharmacology, in *Neurobehavioral Pharmacology* (Thompson T, Dews PB, and Barrett JE eds) pp 275-302, Lawrence Erlbaum Associates, Hillsdale, NJ.

Woolverton WL and Balster RL (1981) Effects of combinations of phencyclidine and pentobarbital on fixed-interval performance in rhesus monkeys. *J Pharmacol Exp Ther* **217:**611-618.

Yoon MH, Choi J, Bae HB, Kim SJ, Chung ST, Jeong SW, Chung SS, Yoo KY, and Jeong CY (2006) Antinociceptive effects and synergistic interaction with morphine of intrathecal metabotropic glutamate receptor 2/3 antagonist in the formalin test of rats. *Neurosci Lett* **394:**222-226.

Yung KK (1998) Localization of glutamate receptors in dorsal horn of rat spinal cord. *Neuroreport* **9:**1639-44.

Zhu J, Luo LY, Li JG, Chen C, and Liu-Chen LY (1997) Activation of the cloned human kappa opioid receptor by agonists enhances [35S] GTPgammaS binding to membranes: determination of potencies and efficacies of ligands. *J Pharmacol Exp Ther* **282:**676–684.

Zimmerman DM, Leander JD, Reel JK, and Hynes MD (1987) Use of β-funaltrexamine to determine μ opioid receptor involvement in the analgesic activity of various opioid ligands. *J Pharmacol Exp Ther* **241:**374-378.