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Title Page

Roles of Mu Opioid Receptors and Nociceptin/Orphanin FQ Peptide Receptors in Buprenorphine-Induced Physiological Responses in Primates

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Running title page

a) Running title:

MOP vs. NOP in Buprenorphine Antinociception

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47 references

Abstract – 245 words

Introduction – 653 words

Discussion – 1239 words

d) Abbreviations:

MOP, mu opioid peptide receptor; NOP, nociception/orphanin FQ peptide receptor; J-113397, (±)-1-[(3R^*,4R^*)-1-(Cyclooctylmethyl)-3-(hydroxymethyl)-4-piperidinyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one; Ro 64-6198, (1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one; SCH 221510, 3-Endo-8-[bis(2-methylphenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octan-3-ol

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Abstract

Buprenorphine is known as a mu opioid peptide (MOP) receptor agonist, but its antinociception was compromised by activation of nociceptin/orphanin FQ peptide (NOP) receptors in rodents. The aim of this study was to investigate the roles of MOP and NOP receptors in regulating buprenorphine-induced physiological responses in primates (rhesus monkeys). Effects of MOP antagonist (naltrexone), NOP antagonist (J-113397), and NOP agonists (Ro 64-6198 and SCH 221510) on buprenorphine were studied in three functional assays for measuring analgesia, respiratory depression, and itch in primates. Over the dose range of 0.01-0.1 mg/kg, buprenorphine dose-dependently produced antinociception, respiratory depression, and itch/scratching responses, and there was a ceiling effect at higher doses, 0.1-1 mg/kg. Naltrexone 0.03 mg/kg produced similar degrees of rightward shifts of buprenorphine's dose-response curves for all three endpoints. Mean pK_B values of naltrexone, 8.1-8.3, confirmed that MOP receptors mainly mediated buprenorphine-induced antinociception, respiratory depression, and itch/scratching. In contrast, J-113397 0.1 mg/kg did not change buprenorphine-induced physiological responses, indicating that there were no functional NOP receptors in buprenorphine-induced effects. More importantly, both NOP agonists, Ro 64-6198 and SCH 221510, enhanced buprenorphine-induced antinociception without respiratory depression and itch/scratching. The dose-addition analysis revealed that buprenorphine in combination with the NOP agonist synergistically produced antinociceptive effects. These findings provided functional evidence that activation of NOP receptors did not attenuate buprenorphine-induced antinociception in primates; instead, co-activation of MOP and NOP receptors produced synergistic antinociception without other side effects. This study strongly supports the therapeutic potential of mixed MOP/NOP agonists as innovative analgesics.

Introduction

Buprenorphine is a mu opioid peptide (MOP) receptor agonist commonly used in clinics. Subutex, trade name of buprenorphine, is used for the transition from opioid withdrawal to the treatment of addiction; when used in an injectable formulation (Buprenex), it is used for analgesia (Rosenblum et al., 2008; Kress, 2009; Pergolizzi et al., 2010). Pharmacological studies indicate that buprenorphine has relatively high binding affinity at MOP, kappa (KOP), and delta opioid (DOP) receptors (Huang et al., 2001), and it has partial agonist activity at MOP receptors measured by both *in vitro* and *in vivo* assays (Cowan et al., 1977; Dum and Herz, 1981; Huang et al., 2001; Clark et al., 2006). Given its favorable safety profile such as the ceiling effect on respiratory depression and other side effects, buprenorphine is considered an effective and realistic option for treating patients for a variety of pain conditions (Walsh et al., 1995; Johnson et al., 2005; Pergolizzi et al., 2010).

The unique pharmacological profile of buprenorphine was further documented by the involvement of nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor. NOP receptor knockout mice showed an enhanced antinociceptive effect produced by buprenorphine, but morphine-induced antinociception was not changed in mice lacking NOP receptors. Following pretreatment with the NOP antagonist J-113397, buprenorphine-induced antinociception was enhanced in wild-type mice, but not in NOP receptor knockout mice (Lutfy et al., 2003). In particular, the ascending portion of the dose-response curve for systemic buprenorphine-induced antinociception was enhanced by the NOP antagonists (Lutfy et al., 2003; Ding and Raffa, 2009; Khroyan et al., 2009). These findings suggest that in mice buprenorphine-induced antinociception is compromised by concomitant activation of NOP receptors. However, ligand-receptor binding studies reveal that

buprenorphine has extremely low binding affinity at NOP versus MOP receptors (i.e., Ki: 285 nM versus 0.08 nM) and the functional assay of G protein activation indicates that buprenorphine is much less potent in stimulating [^{35}S]GTPγS binding to membranes of CHO cells transfected with NOP and MOP receptors with percent stimulation less than half that of MOP receptors (i.e., EC50: 35 nM versus 0.08 nM) (Huang et al., 2001). It is puzzling how buprenorphine activates NOP receptors at the antinociceptive dose range. Nevertheless, it is important to investigate the receptor mechanisms underlying buprenorphine-induced antinociception, particularly in other species such as non-human primates.

Activation of NOP receptors in rodents at the peripheral, spinal, and systemic levels produce pronociception, antinociception, or no effect depending upon the doses and assays (Inoue et al., 1999; Calo' et al., 2000; Jenck et al., 2000). In contrast, in primates following peripheral, spinal, and systemic administration, NOP agonists produce only antinociception regardless of doses and assays applied (Ko, 2004; Ko et al., 2006b, 2009; Ko and Naughton, 2009; Hu et al., 2010). More importantly, there are two independent components, i.e., MOP and NOP receptors, that can equally contribute to antinociceptive effects in primates (Ko et al., 2009; Hu et al., 2010). Given that physiological functions of opioid receptor subtypes are similar between monkeys and humans (Bailey et al., 1993; Palmer et al., 1999; Lee et al., 2007; Ko and Husbands, 2009), pharmacological studies using non-human primates may establish a translational bridge to the therapeutic profiles of drugs in humans. Therefore, it is valuable to pharmacologically investigate buprenorphine-induced physiological responses in primates in order to improve our understanding of receptor mechanisms underlying such effects in humans.

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To date, effects of NOP antagonists and agonists on buprenorphine-induced antinociception in primates are totally unknown. Given that some pharmacological bases of MOP- and NOP-related ligands such as active dose ranges in primates have been established (Ko et al., 2006a,b, 2009; Hu et al., 2010), it would facilitate the study of buprenorphine's receptor mechanisms. Therefore, the aim of this study was to compare effects of MOP and NOP antagonists on buprenorphine-induced antinociception, respiratory depression, and itch/scratching in primates. More importantly, NOP antagonist and agonist were used to elucidate the role of NOP receptors in buprenorphine-induced antinociception.

Materials and Methods

Subjects

Twenty four adult female and male rhesus monkeys (*Macaca mulatta*) ranging in body weight (7.4-12.2 kg) were used. The monkeys were individually housed and their daily dietary intake included approximately 25-30 biscuits (Purina Monkey Chow; Ralston Purina Co., St. Louis, Missouri), fresh fruit, and water ad libitum. For one month prior to the present study, these monkeys were not exposed to any opioid compound. All monkeys had previously been trained in the warm water tail-withdrawal assay and acclimated to being video-recorded in cage. Eighteen monkeys participated in the first two parts of the study, i.e., six subjects were consistently used in a single assay and there were three assays, antinociception, respiratory depression, and itch/scratching responses. The remaining six monkeys were used in the third part of the study for all three assays. The monkeys were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The studies were conducted in accordance with the University Committee on the Use and Care of Animals in the University of Michigan (Ann Arbor, Michigan) and the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (Bethesda, Maryland).

Procedures

Nociceptive responses

The warm water (50°C) tail-withdrawal assay was used to assess thermal antinociceptive effects of the test compound (Ko et al., 2006a). Monkeys were seated in primate restraint chairs, and the lower part of their shaved tails (approximately 15 cm) were immersed in a thermal flask containing water maintained at either 42, 46, or 50°C. 42

and 46°C water were used as non-noxious stimuli whereas 50°C water was used as an acute noxious stimulus. Tail-withdrawal latencies were measured using a computerized timer by an experimenter who was blinded to experimental conditions. If monkeys did not remove their tails within 20 s, the flask was removed and a maximum time of 20 s was recorded. Test sessions began with control determinations at each temperature. Subsequent tail-withdrawal latencies were determined at multiple time points after systemic administration of the test compound.

Respiratory function

The apparatus is similar to that described previously (Butelman et al., 1993). The monkey was seated in a primate restraint chair, enclosed within a sound-attenuating chamber. A rectangular helmet (13.5 x 17.0 x 13.5 cm) was placed over the head of the monkey and sealed around its neck by a latex shield. Gas (either air or a mixture of 5% CO_2 in air) flowed into the helmet and was pumped out at a rate of 8 L/min. The monkeys' breathing produced changes in pressure inside the helmet that were measured with a pressure transducer connected to a polygraph (Grass Model 7). The data were recorded on a polygraph trace and in a microprocessor (IBM PC) through an analog-to-digital converter. The polygraph integrator was connected to a computer, which analyzes the data collected over a 3-min period. The rate of breathing (f, respiratory frequency) was determined directly. The minute volume (V_E), the number of liters of air inspired per min, was determined from the integration of the plethysmograph system. Each test cycle was 30 min, which included the first 23-min exposure to air alone and the remaining 7-min exposure to 5% CO_2 mixed in air. Responses in the first two cycles were averaged as a control value. Subsequent respiratory parameters were determined at multiple time points after intramuscular (i.m.) administration of the test compound.

Scratching responses

Monkeys were recorded in-cage for their scratching behavior, which has been previously associated to an itch sensation following administration of opioid-related ligands (Ko et al., 2004). A scratch was defined as one brief (<1 s) episode of scraping contact of the forepaw or hind paw on the skin surface or other body parts. Each recording session was conducted in 15-min intervals and scored by individuals blinded to experimental conditions after i.m. administration of the test compound.

Experimental Design

The first part of the study was to determine the monkeys' physiological responses to buprenorphine, along with corresponding dose-dependent effects. The dose range, 0.01-1 mg/kg, was selected based on previous human and monkey studies illustrating active dosing conditions (Walker et al., 1995; Zacny et al., 1997; Kishioka et al., 2000; Pergolizzi et al., 2010). Buprenorphine-induced physiological responses were mainly characterized *in vivo* by three distinct measurements, i.e., antinociception, respiratory depression, and itch/scratching activity. These endpoints have been used previously to document the roles of MOP and NOP in regulating physiological functions in monkeys (Butelman et al., 1993; Ko et al., 2004, 2009). Buprenorphine was administered systemically by using a single dosing procedure and each measurement was conducted per 30 min during a 3-hr test session.

The second part of the study was to elucidate the receptor mechanisms underlying buprenorphine-induced physiological responses by using the MOP and NOP antagonists. The dose-response curve of buprenorphine for each endpoint was established by using a cumulative dosing procedure with a 30-min inter-injection interval. A single dose of the

MOP antagonist naltrexone (0.03 mg/kg) or the NOP antagonist J-113397 (0.1 mg/kg) was used based on previous studies, demonstrating that these dosing regimens produced selective, same degree of MOP versus NOP antagonism in monkeys (Ko et al., 2009; Hu et al., 2010). The dose-response curve of buprenorphine for antinociception or itch/scratching was re-determined 15 min after pretreatment with either naltrexone or J-113397. For measuring respiratory depression, the pretreatment time was 30 min due to the setting of 30-min cycles.

The third part of the study was to determine how activation of NOP affected buprenorphine-induced physiological responses. The dose-addition analysis was used to evaluate the drug interaction between buprenorphine and two selective NOP agonists, Ro 64-6198 and SCH 221510 (Jenck et al., 2000; Varty et al., 2008). Both NOP agonists have been studied in primates and their antinociceptive effects were antagonized by the NOP antagonist J-113397, but not by the MOP antagonist naltrexone (Ko et al., 2009; Wladischkin et al., 2012). Initially, dose-response curves for buprenorphine- and NOP agonist-induced antinociception were determined. Depending on the potency of both NOP agonists, dose-response curves for three mixtures of either Ro 64-6198 or SCH 221510 in combination with buprenorphine were re-determined in order to elucidate the nature of the interaction between MOP- and NOP-mediated functions (i.e., sub-additive, additive, or supra-additive effects) (Tallarida, 2000). After drug combination studies, the dose-response curve of buprenorphine alone was re-determined. Dose-response curves for all three endpoints, antinociception, respiratory depression, and itch/scratching, were established to verify the therapeutic profiles of all three mixtures. All experiments including systemic buprenorphine were conducted once per 2-3 weeks.

Data Analysis

Mean values (mean ± S.E.M.) were calculated from individual values for all endpoints. Comparisons were made across all test sessions in the same experiment. Data were analyzed by a two-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple (*post hoc*) comparisons. The criterion for significance was set at $p < 0.05$. For analyzing the dose-response curves for antinociception, individual tail-withdrawal latencies were converted to percentage of maximum possible effect. The formula of the % maximum possible effect is defined as [(test latency – control latency)/(cutoff latency, 20 s – control latency)] x 100. ED50 values were calculated by least-squares regression with the portion of the dose response curves spanning the 50% maximum possible effect. For analyzing the dose-response curves for respiratory depression, ED30 values were calculated due to the ceiling effects of buprenorphine on this endpoint. The 95% confidence limits were also determined. Mean ED30/ED50 values were considered to be significantly different when their 95% confidence limits did not overlap.

For *in vivo* apparent pK_B analysis, a dose ratio was determined for a single dose of the antagonist by using a modified equation, $pK_B = -\log [B/(\text{dose ratio} – 1)]$, where B equals the antagonist dose in moles per kilogram. Mean pK_B values $\pm$ 95% confidence limits were averaged from individual pK_B values for the antagonist. The dose-addition analysis using isobolograms has been widely used to provide a more quantitative evaluation of drug-drug interactions through various endpoints measured in different laboratories (Tallarida 2001; Stevenson et al., 2003; Fischer and Dykstra, 2006; Ko and Husbands, 2009). Statistical evaluation of drug interactions between NOP agonists and buprenorphine was conducted by comparing the experimentally determined ED50 values for each mixture (Zmix) with predicted additive ED50 values (Zadd) that were calculated by

the software developed by Tallarida (2000). Zadd values were calculated individually for

each monkey by using the equation, Zadd = fA + (1-f)B, where A is the ED50 for the NOP

agonist alone, B is the ED50 for buprenorphine alone (Tallarida 2001). The proportion of

the NOP agonist in each mixture was determined by the equation, fA/[fA + (1-f)B]. The

present study investigated effects produced by mixtures with f = 0.25, 0.5, and 0.75,

representing the fractional multiplier for three mixtures of the NOP agonist in combination

with buprenorphine (Tallarida, 2001; Stevenson et al., 2003; Fischer and Dykstra, 2006;

Ko and Husbands, 2009). When f=0.25, the mixture contains a proportion of [A/(A + 3B)]

NOP agonist and a mixture ratio of [(A/B)/3] parts NOP agonist to one-part buprenorphine;

f=0.5 contains a proportion of [A/(A + B)] NOP agonist in the mixture and a mixture ratio of

(A/B) parts NOP agonist to one-part buprenorphine; and f=0.75 contains a proportion of

[A/(A + B/3)] NOP agonist in the mixture and a mixture ratio of [(A/B) x3] parts NOP

agonist to one-part buprenorphine. Mean experimentally determined ED50 values (Zmix)

and predicted additive ED50 values (Zadd) for each mixture were compared with a t-test.

The criterion for significance was set at $p<0.05$.

Drugs

Naltrexone HCl (National Institute on Drug Abuse, Bethesda, Maryland) and J-113397

(Tocris Bioscience, Ellisville, Missouri) were dissolved in sterile water. Ro 64-6198 (Amgen

Inc., Thousand Oaks, California) and SCH 221510 (Tocris Bioscience, Ellisville, Missouri)

were dissolved in a solution of DMSO/Tween80/sterile water in a ratio of 1:1:8.

Buprenorphine HCl (National Institute on Drug Abuse, Bethesda, Maryland) was dissolved

in sterile water with the addition of a few drops of lactic acid. Doses are presented in the

compound forms listed above. For systemic administration, all compounds were

administered at a volume of 0.1 mL/kg.

Results

Figure 1 illustrates the time course and dose dependency of buprenorphine-induced antinociceptive effects against an acute nociceptive stimulus, $50^{\circ}C$ water, in primates. Following subcutaneous administration, buprenorphine produced antinociception in both dose- [$F_{(5,25)}=30.7$; $p<0.05$] and time-dependent [$F_{(5,25)}=13.4$; $p<0.05$] manners. Over low doses, 0.03-0.1 mg/kg, buprenorphine-induced antinociception peaked at the first observation period (i.e., 30 min after administration), then decreased at 2-3 hr after administration (Figure 1A). Over higher doses, 0.3-1 mg/kg, similar magnitudes and durations of buprenorphine-induced antinociception were observed, i.e., higher doses of buprenorphine did not further increase antinociceptive effects under this condition (Figure 1B).

Figure 2 illustrates the time course and dose dependency of buprenorphine-induced respiratory depression in primates breathing 5% CO_2 mixed in air. As the magnitudes and durations of buprenorphine-induced respiratory depression were similar between cycles of air versus 5% CO_2 in air, only data during the cycle of 5% CO_2 in air are presented herein. Following intramuscular administration, buprenorphine-induced respiratory depression was dose-dependently manifested by both parameters, f [$F_{(5,25)}=10.1$; $p<0.05$] and V_E $_{(5,25)}=15.0$; $p<0.05$] (Figures 2A & 2B). There was no time-dependency in changes of both parameters, f [$F_{(5,25)}=1.4$; $p<0.05$] and V_E [$_{(5,25)}=0.6$; $p>0.05$] during the first 3 hr after administration. Over low doses, 0.03-0.1 mg/kg, buprenorphine-induced respiratory depression peaked at 30 min and continued throughout the 3-hr test period (Figures 2A & 2B). Over higher doses, 0.3-1 mg/kg, buprenorphine produced similar effects as 0.1 mg/kg did in suppressing respiratory parameters, f and V_E (Figures 2C & 2D).

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Based on data obtained in Figures 1 and 2, multiple doses of buprenorphine (0.01-0.3 mg/kg) were selected to study the effects of buprenorphine on eliciting itch/scratching responses. Figure 3 shows the duration and magnitude of buprenorphine-induced scratching activity. Following intramuscular administration, buprenorphine dose-dependently elicited scratching responses [$F_{(4,20)}=9.7$; $p<0.05$]. Effects of buprenorphine (0.1-0.3 mg/kg) peaked at the first observation period (i.e., 30 min after administration) and continued throughout the 3-hr observation period.

Figure 4 compares the antagonist effects of the MOP antagonist naltrexone and NOP antagonist J-113397 on buprenorphine-induced antinociceptive effects. Following 15 min pretreatment time, a single dose of subcutaneous naltrexone 0.03 mg/kg produced a large rightward shift of the dose-response curve of buprenorphine-induced antinociception and its mean naltrexone pK_B value was 8.2. In contrast, subcutaneous pretreatment with J-113397 0.1 mg/kg failed to block buprenorphine-induced antinociception. The ED50 value of buprenorphine dose-response for vehicle pretreatment (0.02 mg/kg) was similar to that for J-113397 pretreatment (0.03 mg/kg) (see details in Table 1).

Figure 5 compares the effects of the MOP antagonist naltrexone and NOP antagonist J-113397 on blocking the respiratory depressant effects of buprenorphine. Following 30 min pretreatment time, intramuscular naltrexone 0.03 mg/kg produced a large rightward shift of the dose-response curve of buprenorphine-induced respiratory depression in the V_E parameter and its mean naltrexone pK_B value was 8.1. In contrast, J-113397 0.1 mg/kg did not change the dose-response curves of buprenorphine for both f and V_E parameters. The ED30 value of buprenorphine dose-response for vehicle pretreatment (0.032 mg/kg) was similar to that for J-113397 pretreatment (0.045 mg/kg) (Table 1). As noted, there were no changes in both f and V_E parameters following

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administration of either naltrexone or J-113397 as compared to the control value under this condition (data not shown).

Figure 6 compares the antagonist effects of the MOP antagonist naltrexone and NOP antagonist J-113397 on buprenorphine-induced scratching responses. Following 15 min pretreatment time, intramuscular naltrexone 0.03 mg/kg produced a large rightward shift of the dose-response curve of buprenorphine-induced scratching and its mean naltrexone pK_B value was 8.3. In contrast, J-113397 0.1 mg/kg failed to block buprenorphine-induced scratching. The ED50 value of buprenorphine dose-response for vehicle pretreatment (0.015 mg/kg) was similar to that for J-113397 pretreatment (0.016 mg/kg) (Table 1).

Figure 7 illustrates the effects of buprenorphine alone or in combination with either Ro 64-6198 or SCH 221510. Addition of each NOP agonist dose-dependently produced leftward shifts in the dose-response curve of buprenorphine-induced antinociception (Figures 7A & 7B). In contrast, when the ratio of the NOP agonist versus buprenorphine increased, the combination of each NOP agonist with buprenorphine did not significantly produce respiratory depression (Figures 7C & 7D) or elicit itch/scratching responses (Figures 7E & 7F) along with the corresponding doses that produced full antinociceptive effects.

Figure 8 displays isobolograms for both mixtures of Ro 64-6198+buprenorphine (Figure 8A) and SCH 221510+buprenorphine (Figure 8B). The experimentally determined ED50 values (Zmix) and predicted additive ED50 values (Zadd) are shown in Table 2. The statistical analysis confirmed that Zmix values were significantly different from Zadd values for each mixture, indicating that there was a synergistic interaction for antinociceptive

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effects produced by buprenorphine in combination with either Ro 64-6198 or SCH 221510

in primates.

Discussion

The first part of the study showed that systemic administration of buprenorphine dose-dependently produced antinociception, respiratory depression, and itch/scratching at the dose range of 0.01-0.1 mg/kg (Figures 1-3). Up to a 10-fold higher dose, 1 mg/kg of buprenorphine retained its antinociception without aggravating respiratory depression. This ceiling effect of buprenorphine-induced respiratory depression clearly supports its wide safety margin in clinical use (Rosenblum et al., 2008; Kress, 2009; Pergolizzi et al., 2010). Buprenorphine has been estimated to be 30 to 50 times more potent than morphine in humans (Jasinski et al., 1978; Kress et al., 2009; Pergolizzi et al., 2010). This potency difference between systemic buprenorphine and morphine maintains the same ratio in the primate nociceptive assay, based on either ED50 values (0.02-0.04 versus 0.9 mg/kg) or the minimum doses (0.1 versus 3 mg/kg) producing full antinociception (Walker et al., 1995; Lee et al., 2007; Ko and Husbands, 2009). More importantly, a single dose of buprenorphine 0.1 mg/kg in primates produced antinociceptive effects accompanied by a mild to moderate degree of respiratory depression and itch, a similar profile as reported in human studies (Zacny et al., 1997; Kress et al., 2009; Pergolizzi et al., 2010). These findings indicate that the therapeutic profile of buprenorphine in humans can be manifested and studied in different physiological functional assays in non-human primates.

The second part of the study demonstrated that there were distinct antagonist effects between MOP and NOP antagonists on buprenorphine-induced physiological responses (Figures 4-6). Pretreatment with the MOP antagonist naltrexone 0.03 mg/kg produced large rightward shifts (~20-30 fold) of buprenorphine's dose-response curves for antinociception, respiratory depression, and itch/scratching. The mean naltrexone pK_B values for these endpoints ranged between 8.1 and 8.3. In previous studies using

primates, this dose of naltrexone has been shown to selectively block MOP agonist-induced effects and it has similar naltrexone pK_B values ranging from 8.2 to 8.6 (Ko et al., 2004, 2006a,b, 2009). These findings clearly suggest that buprenorphine-induced antinociception, respiratory depression, and itch/scratching in primates are mainly mediated by MOP receptors. Such a conclusion may be expected since buprenorphine has relatively high binding affinity and measurable intrinsic activity at MOP receptors (Traynor and Nahorski, 1995; Huang et al., 2001; Zaveri et al., 2001; Clark et al., 2006), and rodent studies also indicate that buprenorphine-induced antinociception is mainly mediated by MOP receptors (Kogel et al., 2005; Yamamoto et al., 2006).

Unlike findings in rodent studies (Lutfy et al., 2003; Ding and Raffa, 2009; Khroyan et al., 2009), the NOP antagonist J-113397 did not enhance buprenorphine-induced antinociception in primates. A single dose 0.1 mg/kg of J-113397 has been demonstrated to selectively block NOP agonist-induced effects without interfering with MOP receptor-mediated effects (Ko et al., 2006b, 2009; Hu et al., 2010; Podlesnik et al., 2011). In particular, this dosing regimen produced approximately a 30-fold rightward shift of the dose-response curve for the NOP agonist Ro 64-6198-induced antinociception in primates (Ko et al., 2009). Pretreatment with the same dose of J-113397 did not produce any shifts of buprenorphine's dose-response curves, indicating that there are no detectable functional NOP receptors involved in buprenorphine-induced physiological responses in primates under this condition.

Buprenorphine has relatively low binding affinity at NOP receptors; its Ki values range from 77 to 285 nM and its binding selectivity for MOP versus NOP receptors varies from 50- to 3500-fold (Huang et al., 2001; Zaveri et al., 2001; Spagnolo et al., 2008; Khroyan et al., 2009). In the functional assay of $[^{35}S]GTP\gamma S$ binding for NOP receptors,

buprenorphine produced no stimulation (Spagnolo et al., 2008) or mild to moderate stimulation (16-60% compared to N/OFQ) as a partial agonist action (Huang et al., 2001; Khroyan et al., 2009). It seems unlikely that buprenorphine activates NOP receptors within the antinociceptive dose range. There are several newly developed compounds such as SR16435 and BU08028 that have high binding affinity for both MOP and NOP receptors (i.e., Ki values: 2-8 nM) with 20-50% of [^{35}S]GTPγS binding stimulation (Spagnolo et al., 2008; Khroyan et al., 2009, 2011). Interestingly, in rodent studies pretreatment with the NOP antagonist enhanced all of the ascending portions of the dose-response curves for antinociception produced by SR16435, BU08028, and buprenorphine (Khroyan et al., 2009, 2011). These findings may suggest that the binding "*selectivity*" for MOP versus NOP receptors (i.e., 50- to 3500-fold versus no selectivity) does not prevent the compound's susceptibility to the NOP antagonism in rodents. It will be important to further investigate whether compounds without the MOP/NOP binding selectivity (i.e., mixed MOP/NOP agonists) have different therapeutic profiles compared to buprenorphine in primates.

The third part of the study examined effects of the NOP agonists on buprenorphine-induced physiological functions by using the dose-addition analysis and isobolograms (Figures 7-8). What is exciting is that the combined administration of buprenorphine with either Ro 64-6198 or SCH 221510 resulted in synergistic antinociceptive effects in primates. These results provide novel functional evidence that activation of NOP receptors does not attenuate MOP agonist-induced antinociception; instead, there is a potentiated antinociception produced by co-activation of MOP and NOP receptors at the systemic level in primates. What is more stimulating is that by increasing the ratio of the NOP agonist in combination with buprenorphine, the specific mixture produced full antinociception without respiratory depression and itch/scratching (e.g., dosing conditions of filled squares and

circles presented in Figure 7). When N/OFQ was combined with a single dose of intrathecal morphine, this combination dose-dependently enhanced morphine-induced antinociception without compromising primates' motor function (Ko and Naughton, 2009). When an inactive dose of the NOP agonist UFP-112 was combined intrathecally with an inactive dose of morphine, such a mixture significantly produced antinociception against capsaicin-induced allodynia (Hu et al., 2010). Considering that simultaneous activation of two receptors to a small degree produces therapeutic effects with less side effects (i.e., a wider therapeutic window), these findings strongly indicate that mixed MOP/NOP agonists may represent a novel strategy for pain management.

Collectively, this study demonstrates that MOP receptors mainly mediate buprenorphine-induced antinociception, respiratory depression, and itch/scratching in primates. Unlike rodent studies showing that the NOP antagonists enhanced buprenorphine-induced antinociception (Lutfy et al., 2003; Ding and Raffa, 2009; Khroyan et al., 2009), the NOP antagonist J-113397 did not change buprenorphine-induced antinociception in primates, indicating that NOP receptors are not involved in buprenorphine-induced physiological responses in primates. More importantly, it is the NOP "*agonists*" that actually potentiated MOP-mediated antinociception in primates. Physiological functions of opioid receptor subtypes and the pharmacological profiles of synthetic opioid-related compounds may vary between rodents and primates. For example, a buprenorphine-like compound, BU72, was characterized as a MOP agonist with a wide therapeutic window in rodents, but BU72 was very potent in producing respiratory depression in primates and it displayed a very narrow window between antinociceptive doses and doses producing side effects (Neilan et al., 2004). The present study is the first to provide functional evidence that systemic co-administration of MOP and NOP agonists

produced synergistic antinociceptive effects without other side effects in primates. Given

that NOP agonists have low abuse liability and/or anti-addiction property (Ko et al., 2009;

Khroyan et al., 2011; Zaveri, 2011), it is worth developing bifunctional MOP/NOP agonists

as analgesics or anti-addiction drugs (Spagnolo et al., 2008; Khroyan et al., 2011; Zaveri,

2011; Cami-Kobeci et al., 2011). If NOP agonists have as yet undescribed side effects that

limit their use as analgesics, adding them to MOP agonists may allow doses of both drugs

to be reduced, analgesia enhanced, and side-effects of both reduced. In other words, by

reserving most functional receptor pools, simultaneous activation of two receptor

components to a small degree may produce desirable therapeutic effects with less side

effects. Not only producing a wider therapeutic window, this approach may also provide a

reduced tolerance liability when two receptors are repeatedly activated due to a large

reservoir for both receptor populations. Future studies are warranted to compare the rates

and degrees of tolerance development following chronic administration of bifunctional

MOP/NOP agonists versus selective agonists targeting one receptor and to validate their

therapeutic potential in primate models.

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Acknowledgments

Authorship Contributions

Participated in research design: Kyle and Ko.

Conducted experiments: Cremeans and Gruley.

Performed data analysis: Cremeans, Gruley, and Ko.

Wrote or contributed to the writing of the manuscript: Cremeans, Gruley, Kyle, and Ko.

Other: Kyle and Ko acquired funding for the research.

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Footnotes

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Legends for figures

Figure 1.

Antinociceptive effects of subcutaneously administered buprenorphine over a wide dose range against an acute noxious stimulus $50^{\circ}C$ water in primates. Each data point represents a mean ± S.E.M. (n=6). The asterisk represents a significant difference from the vehicle condition from the time point 30 min to the corresponding time point for each dose (∗, $p < 0.05$). The same data of buprenorphine 0.1 mg/kg are presented in both panels 1A (●) & 1B (▼) for comparison with other doses.

Figure 2.

Respiratory depressant effects of intramuscularly administered buprenorphine over a wide dose range in primates breathing air mixed with 5% CO_2. Each data point represents a mean ± S.E.M. (n=6). The asterisk represents a significant difference from the vehicle condition for all time points (∗, $p < 0.05$). The same data of buprenorphine 0.1 mg/kg are presented in both top (2A & 2B, ●) and bottom (2C & 2D, ▼) panels for comparison with other doses.

Figure 3.

Itch/scratching eliciting effects of intramuscularly administered buprenorphine over a wide dose range in primates. Each data point represents a mean ± S.E.M. (n=6). The asterisk represents a significant difference from the vehicle condition from the time point 30 min to the corresponding time point for each dose (∗, $p < 0.05$).

Figure 4.

Effects of MOP and NOP antagonists on buprenorphine-induced antinociception in primates. A MOP antagonist naltrexone (0.03 mg/kg) or a NOP antagonist J-113397 (0.1

mg/kg) was administered subcutaneously 15 min before determination of buprenorphine's

dose-response curve. Each data point represents a mean ± S.E.M. (n=6).

Figure 5.

Effects of MOP and NOP antagonists on buprenorphine-induced respiratory depression in

primates. A single dose of the MOP antagonist naltrexone (0.03 mg/kg) or the NOP

antagonist J-113397 (0.1 mg/kg) was administered intramuscularly 30 min before

administration of the first dose of buprenorphine. Data represent the changes of both

parameters, f (panel 5A) and V_E (panel 5B), in primates breathing air mixed with 5% CO_2.

Each data point represents a mean ± S.E.M. (n=6).

Figure 6.

Effects of MOP and NOP antagonists on buprenorphine-induced itch/scratching activity in

primates. A single dose of the MOP antagonist naltrexone (0.03 mg/kg) or the NOP

antagonist J-113397 (0.1 mg/kg) was administered intramuscularly 15 min before

determination of buprenorphine's dose-response curve. Each data point represents a

mean ± S.E.M. (n=6).

Figure 7.

Effects of the NOP agonists, Ro 64-6198 and SCH 221510, in combination with

buprenorphine on antinociception, respiratory depression, and itch/scratching responses.

Top panels (7A & 7B): dose-response curves for antinociception produced by

buprenorphine alone and in mixtures with either Ro 64-6198 or SCH 221510. Middle

panels (7C & 7D): dose-response curves for respiratory depression produced by

buprenorphine alone and in same dose combination, as presented in 7A & 7B, with each

NOP agonist. Bottom panels (7E & 7F): dose-response curves for itch/scratching

responses elicited by buprenorphine alone or in the same dose combination, as presented

in 7A & 7B, with each NOP agonist. All drugs were administered intramuscularly in the

same subjects. Each data point represents a mean ± S.E.M. (n=6). The asterisk

represents a significant difference from the dosing condition of buprenorphine 0.003 mg/kg

alone (∗, $p < 0.05$).

Figure 8.

Effects of the NOP agonists, Ro 64-6198 and SCH 221510, in combination with

buprenorphine on antinociceptive effects against 50°C water. Isobolograms for the mixture

of buprenorphine- with either Ro 64-6198-induced antinociception (8A) or SCH 221510-

induced antinociception (8B) were displayed. . Each data point represents a mean ±

S.E.M. (n=6). See Table 2 and the *Data Analysis* section for other details.

Table 1

Antagonist studies of systemic buprenorphine-induced physiological responses in primates

	Vehicle + Buprenorphine	J-113397 + Buprenorphine	Naltrexone + Buprenorphine		
	ED50 (95% CL) mg/kg	ED50 (95% CL) mg/kg	ED50 (95% CL) mg/kg	Mean Dose Ratio [#]	Mean pK_B (95% CL) [#]
Antinociception	0.020 (0.008-0.051)	0.030 (0.014-0.066)	0.34 (0.14-0.79) ∗	17.7	8.2 (8.0-8.4)
Itch/Scratching	0.015 (0.008-0.027)	0.016 (0.006-0.042)	0.31 (0.14-0.71) ∗	27.2	8.3 (7.8-8.7)
	ED30 (95% CL) mg/kg	ED30 (95% CL) mg/kg	ED30 (95% CL) mg/kg		
Respiratory Depression(V_E)	0.032 (0.012-0.083)	0.045 (0.017-0.12)	0.43 (0.17-1.11) ∗	22.2	8.1 (7.6-8.6)

∗ An ED50 value is significantly different from the ED50 value determined in the dosing

condition, Vehicle + Buprenorphine (p<0.05).

Both mean dose ratio and pK_B value were determined for the dosing condition,

Naltrexone + Buprenorphine (n=6). See Figures 4-6 for other details.

Table 2A

ED50 values (mg/kg) for agonists alone in the primate antinociceptive assay.

Agonist	ED50 (95% C.L.)
Buprenorphine[1]	0.044 (0.037-0.055)
Buprenorphine[2]	0.039 (0.031-0.052)
Ro 64-6198	0.014 (0.012-0.016)
SCH 221510	0.0047 (0.0041-0.0054)

[1] The ED50 value of buprenorphine was determined before drug combinations.

[2] The ED50 value of buprenorphine was determined after drug combinations.

Table 2B

Experimentally determined ED50 values (Zmix) and predicted additive ED50 values (Zadd) of mixtures of NOP agonists administered in combination with buprenorphine in the primate antinociceptive assay.

	Zmix (95% C.L.)	Zadd (95% C.L.)
Ro 64-6198 + Buprenorphine		
0.1:1, Ro 64-6198:Buprenorphine	0.018 (0.017-0.019)*	0.038 (0.031-0.045)
0.3:1, Ro 64-6198:Buprenorphine	0.006 (0.004-0.008)*	0.030 (0.025-0.035)
1:1, Ro 64-6198:Buprenorphine	0.003 (0.0027-0.0033)*	0.022 (0.019-0.026)
SCH 221510 + Buprenorphine		
0.03:1, SCH 221510:Buprenorphine	0.016 (0.015-0.018)*	0.035 (0.029-0.043)
0.1:1, SCH 221510:Buprenorphine	0.007 (0.006-0.008)*	0.025 (0.021-0.030)
0.3:1, SCH 221510:Buprenorphine	0.001 (0.0007-0.0019)*	0.015 (0.012-0.018)

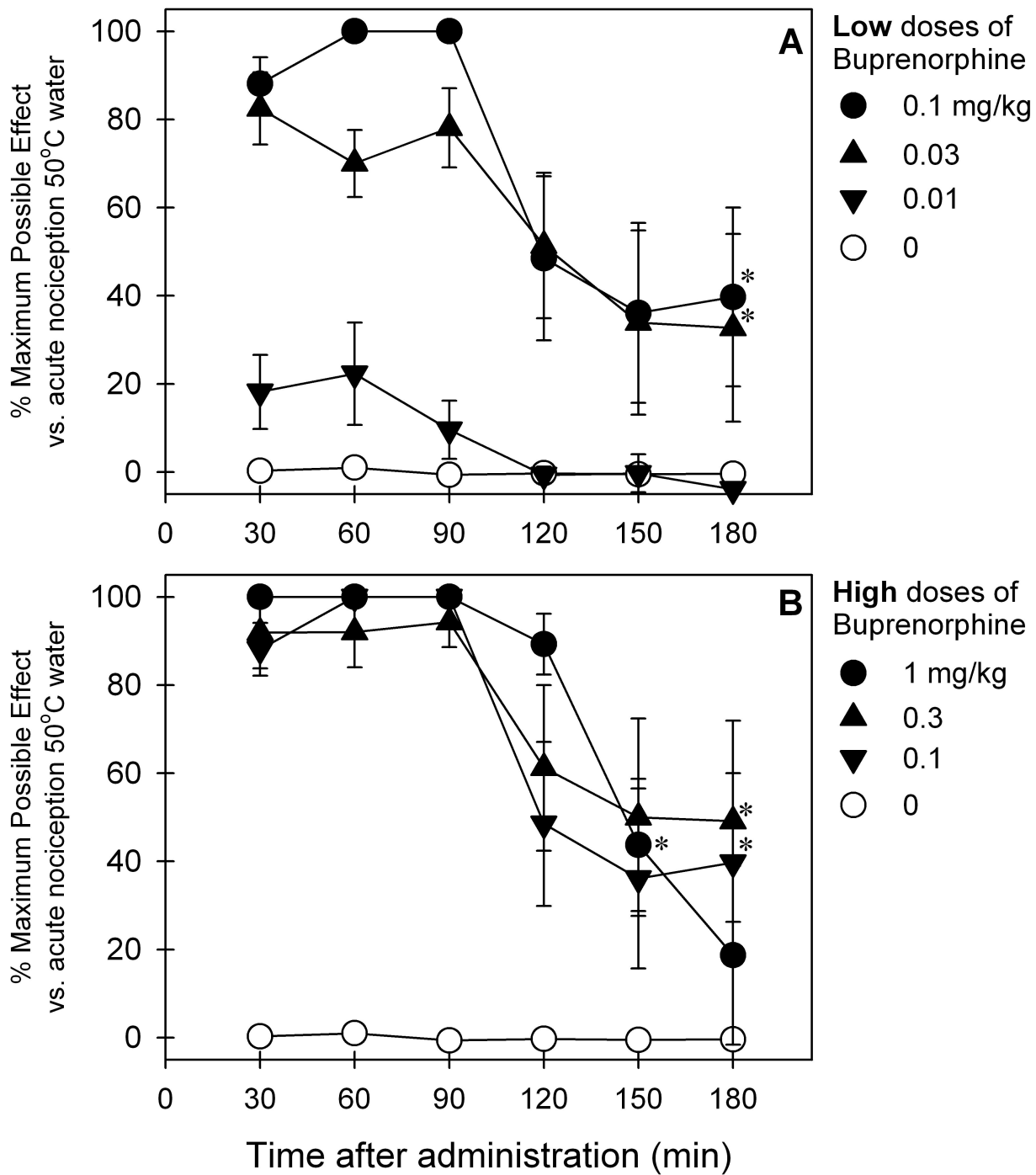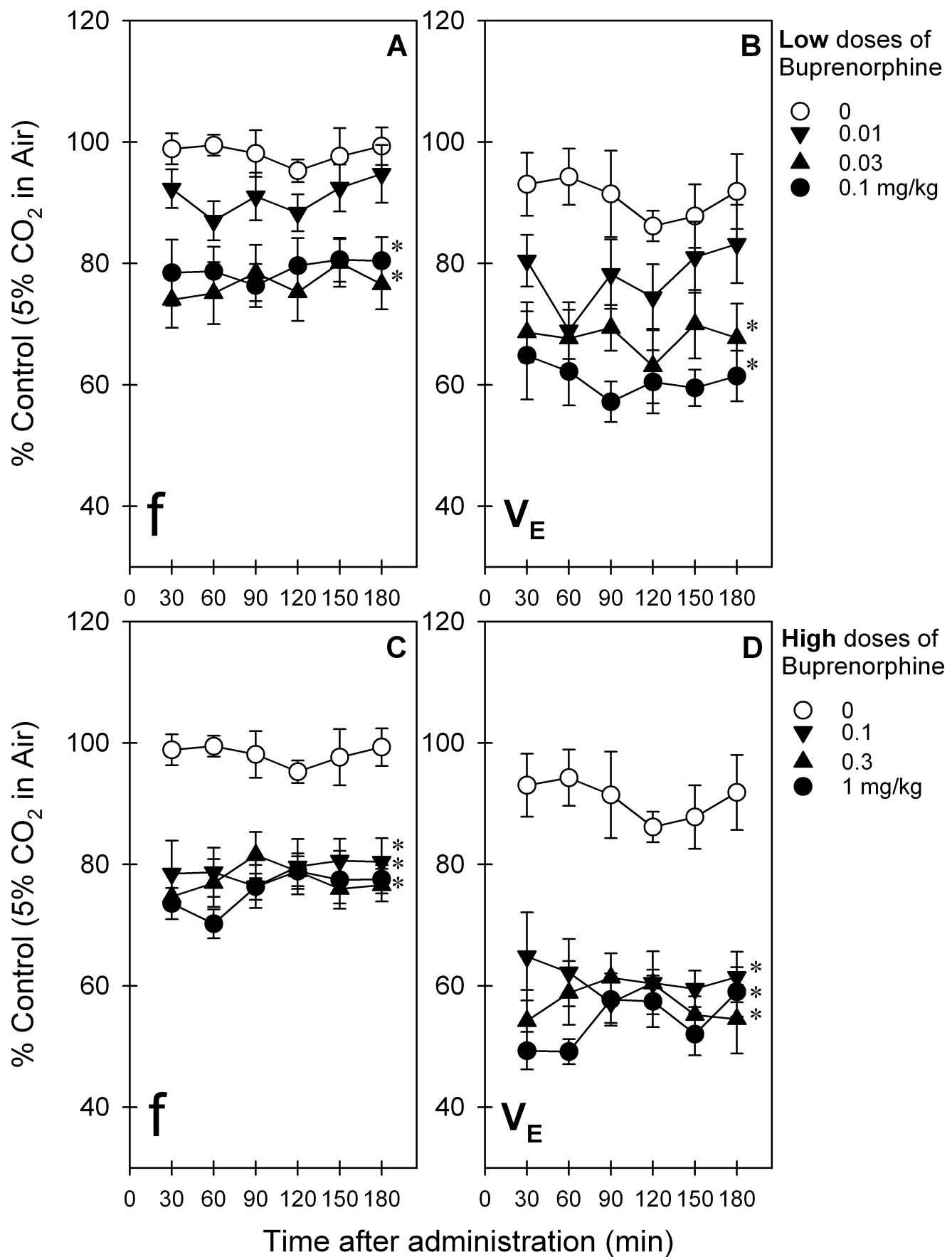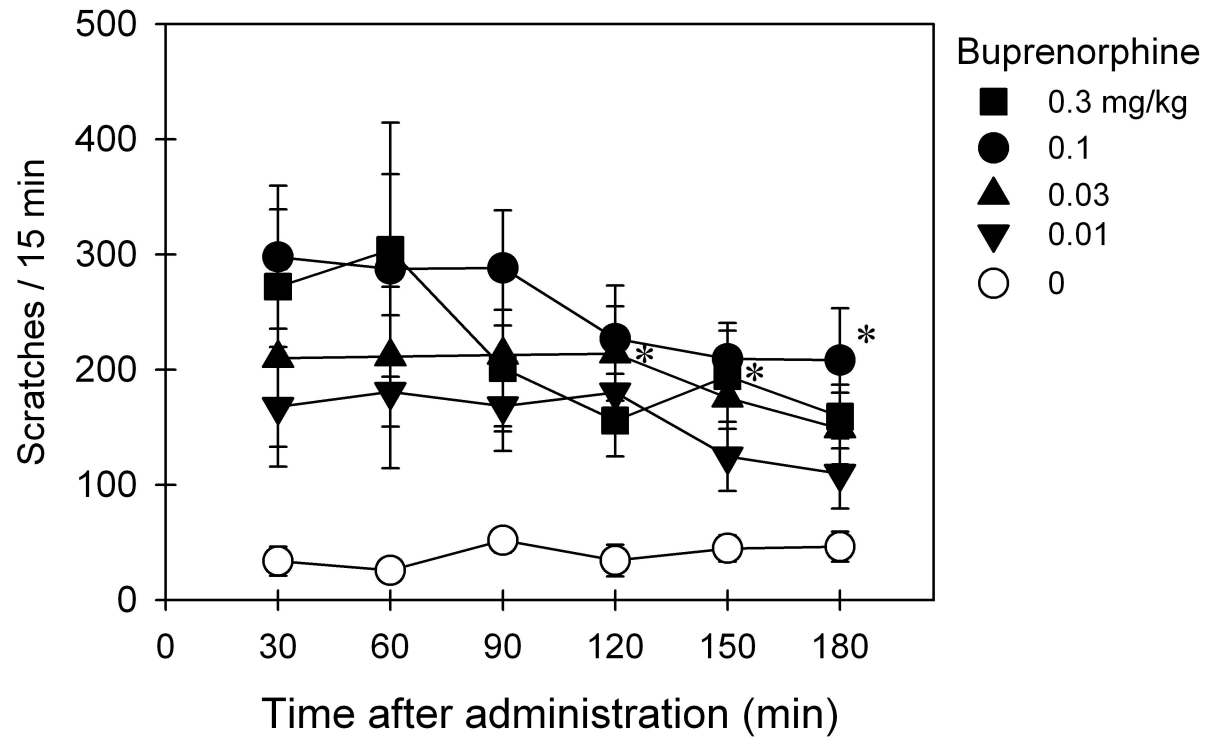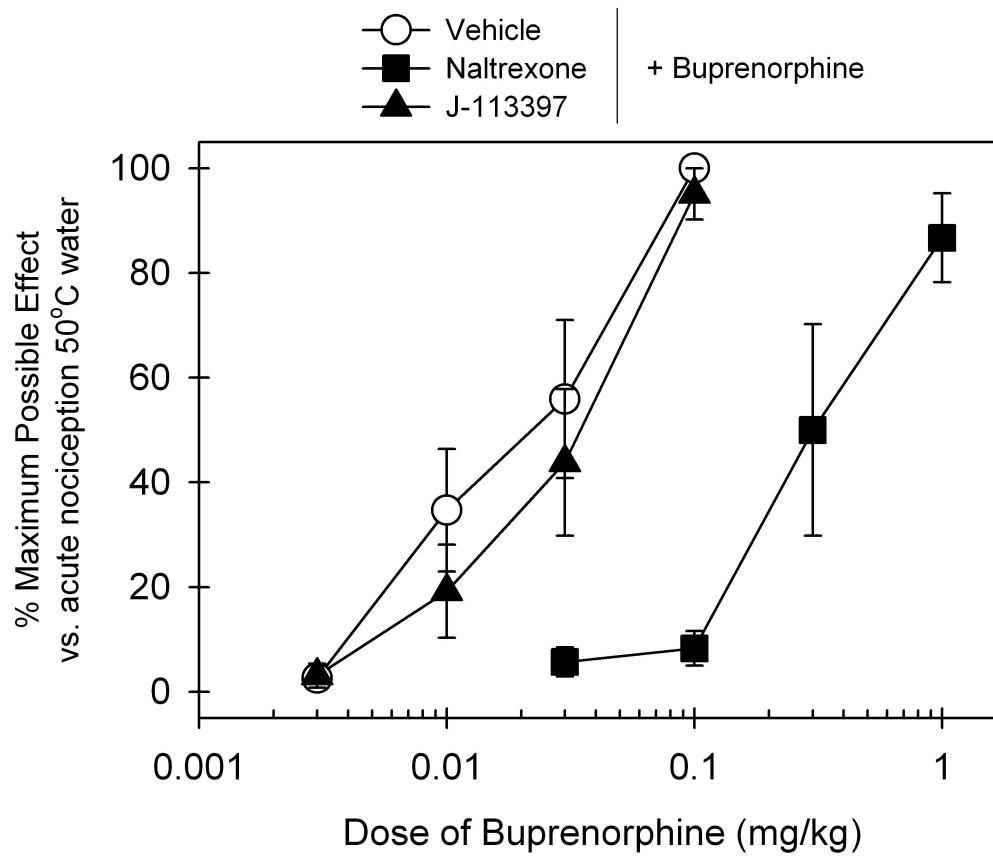* An experimental ED50 value (mg/kg) is significantly different from the predicted additive ED50 value ($p < 0.05$).
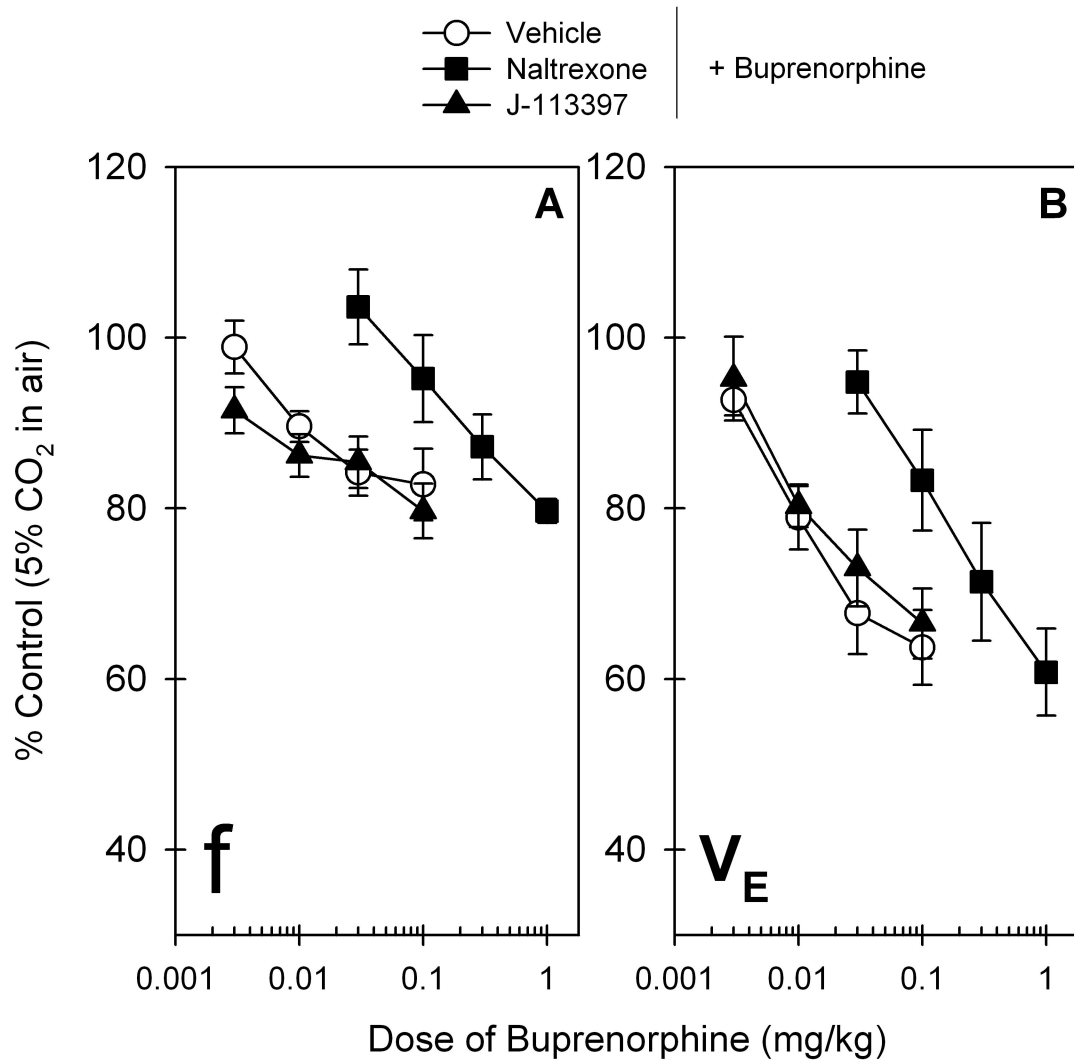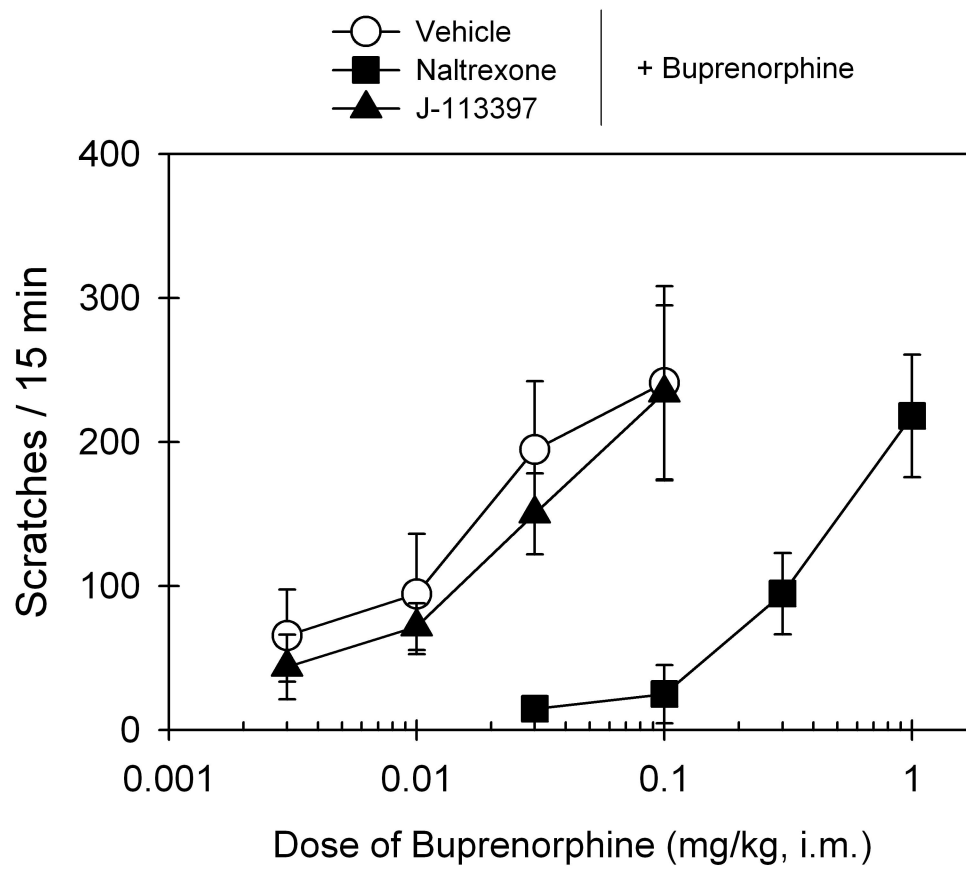
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8