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Title: The Effects of Morphine, Baclofen, and Buspirone Alone and in Combination on Schedule-Controlled Responding and Hot Plate Antinociception in Rats

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Abstract

Better therapeutic options are needed for pain. Baclofen, buspirone, and morphine are characterized as having analgesic properties. However, little is known about potential interactions between analgesic effects of these drugs when combined. Further, it is not known if the magnitude of these potential interactions will be similar for all drug effects. Thus, we tested the effects of these drugs alone and in combination for their capacity to produce thermal antinociception and to decrease food-maintained responding. Four male and four female Sprague-Dawley rats responded for food under a fixed-ratio 10 schedule; afterward they were immediately placed on a 52˚C hot plate. Morphine, baclofen, and buspirone were examined alone and in 1:1 combinations, based upon ED_{50} values. Morphine and baclofen effects were evaluated with the opioid antagonist, naltrexone, and the $GABA_B$ antagonist CGP34358, respectively. Morphine, baclofen, and buspirone dose-dependently decreased operant responding, with the calculated ED_{50} values being 7.09, 3.42, and 0.57 mg/kg, respectively. The respective antinociception ED_{50} values were 16.15, 8.75, and 2.20 mg/kg. Analysis of 1:1 combinations showed the effects of morphine plus baclofen to decrease schedule-controlled responding and to produce thermal antinociception were synergistic. Effects of morphine plus buspirone and baclofen plus buspirone to decrease schedule-controlled responding were additive. Effects of the two combinations to produce thermal antinociception were synergistic. Naltrexone and CGP34358 antagonized the effects of morphine and baclofen, respectively. Synergistic antinociceptive effects, in conjunction with additive effects on food-maintained responding highlights the therapeutic utility of opioid and non-opioid drug combinations.

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Abbreviations:

analysis of variance (ANOVA); Association for Assessment and Accreditation of Laboratory

Animal Care, (AAALAC); confidence limits, (CL); effective dose, (ED); fixed ratio, (FR); Food

and Drug Administration, (FDA); intraperitoneal(ly), (i.p.); percent maximum possible effect, (%

MPE); serotonin, (5-HT); standard error of the mean, (SEM); γ-aminobutyric acid, (GABA)

Introduction

Currently the United States is in the midst of an opioid overdose epidemic. It is likely that opioids will continue to be needed for treating pain for the foreseeable future, especially severe acute pain and cancer pain (Fields, 2011). The low dose combination of drugs from different pharmacological classes, including opioids, is a recognized strategy to circumvent potential untoward side effects (Wilkerson *et al.,* 2016; 2017). Although there is considerable interest in the development of novel therapeutics to treat pain, with a keen interest on minimizing drug abuse liability, the clinical fruition of these alternatives takes many years, often with high costs that are passed down to the patient. Hence, there is notable interest in repurposing drugs that are already U.S. Food and Drug Administration (FDA)-approved for new and innovative uses (Corsello *et al*., 2017). Thus, there may be suitable drugs already available that may lessen the burden of opioid use for pain management by producing stable and adequate pain relief with lessened side-effect profiles when combined with other analgesics such as opioids.

Baclofen, a γ-aminobutyric acid (GABA)$_B$ receptor agonist, is FDA-approved with prescribing indications for muscle spasticity and pain (Sadiq & Poopatana, 2007). In previous preclinical studies baclofen has been found to produce thermal antinociception and to reverse pain-evoked behaviors, such as allodynia, exhibited from neuropathic pain models (Hwang & Yaksh, 1997; Salte *et al.,* 2016; Zemoura *et al.,* 2016; Deseure & Hans 2017). However, clinically, baclofen is often associated with drowsiness, dizziness, nausea, vomiting, and in some cases, seizures. Thus, the use of baclofen as a monotherapy for pain is limited by its side-effect profile. Buspirone can produce effects at numerous receptors and has been found to act as a serotonin (5-HT)$_{1A}$ receptor agonist, as well as a dopamine D_2, D_3, and D_4 antagonist. Buspirone is FDA-approved to treat anxiety as well as depression and has been found in preclinical models

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to produce thermal antinociception (Korneyev & Seredenin, 1993). Morphine, a mu-kappa opioid receptor agonist is a well-known analgesic, but has severe clinical side-effects such as constipation, respiratory depression and abuse liability (Fields, 2011; Wilkerson *et al.,* 2016; 2017).

Previous results suggest that the addition of baclofen to morphine may enhance morphine-induced thermal antinociception in mice and ferrets (Suzuki *et al.,* 2005). Likewise, the combination of buspirone and morphine was found to produce additive thermal antinociceptive effects in non-human primates (Li *et al.,* 2011). Additionally, the addition of buspirone to morphine was found to increase morphine-induced thermal antinociception in rats (Haleem & Nawaz, 2017). Here we sought to systematically examine respective pharmacological interactions and to quantify the potency of these drugs alone and in combination, based upon their behavior-modifying effects. Further, it is not known if the magnitude of these potential interactions will be the same or different for all drug effects. Either synergistic or additive antinociceptive effects in conjunction with either additive or sub-additive behaviorally disruptive effects, respectively, may warrant further development of specific drug combinations as pain therapeutics. Thus, differential behavior-modifying effects in this manner may produce an ideal therapeutic window which would allow for the therapeutic use of drug combinations with minimal adverse effects (Foucquier & Guedj, 2015).

One way to examine drugs preclinically for antinociceptive effects is via the hot plate test. The hot plate test specifically examines reflexive pain transmission and is mediated primarily by supra-spinal pain processing (Eddy & Leimbach, 1953). Further drug-induced disruptions in behavior can be assessed through measurement of schedule-controlled operant responding for food, which is sensitive to drug-induced disruptions in behavior from numerous

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drug classes that act in the central nervous system (McMahon & France, 2002; Cunningham & McMahon, 2010). Thus, the present study tested the effects of these drugs alone and in combination in rats for their capacity to produce thermal antinociception and to decrease schedule-controlled responding for food. Another goal of the present study was to examine the extent by which the effects of morphine drug combinations (i.e, morphine plus baclofen; morphine plus buspirone) are mediated by opioid receptors. This was accomplished by combining the opioid receptor antagonist naltrexone with morphine by itself, as well as with the morphine drug combinations. In addition to opioid receptor involvement, we also sought to examine the extent that GABA$_B$ receptors mediate baclofen drug combination effects (i.e., baclofen plus morphine; baclofen plus buspirone). This was likewise accomplished by combining the GABA$_B$ receptor antagonist CGP35348 with baclofen by itself, as well as with the baclofen drug combinations.

Materials and Methods

Animals

Adult male (n=4) and female (n=4) Sprague Dawley rats (247-279 gram upon arrival, Jackson Laboratory, Charleston, SC) were singly housed in a temperature- (20–22 °C), humidity- (55 ± 10 %), and light-controlled (12 hour light/dark; lights on at 0600 hours) facility that was approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Water was available ad libitum. Rats were food restricted to 90% of their free-feeding body weight, with access to food (Dustless Precision Pellets Grain-Based Rodent Diet, Bio-Serv, Frenchtown, NJ) 30 min following daily experimental sessions, as well as to food during experimental sessions as described below. Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.,* 2010; McGrath *et al.,* 2010).

Drugs

Morphine sulfate pentahydrate (morphine) and naltrexone hydrochloride (naltrexone) were obtained from the National Institute on Drug Abuse Research Technology Branch (Rockville, MD). Baclofen and buspirone were obtained from Sigma-Aldrich (St. Louis, MO). CGP35348 was synthesized as described (Froestl *et al.,* 1995). All drugs were dissolved in sterile saline, and sterile saline served as the vehicle in all experiments. Drugs and vehicle were administered intraperitoneally (i.p.).

Schedule-Controlled Responding

Apparatus

Operant conditioning chambers were 25 cm long, 31 cm high and 25 cm wide, as commercially supplied by Med Associates (Fairfax, VT). Each chamber was equipped with two centrally-mounted, 5 cm-long levers located 9 cm from the chamber floor and 3 cm from either wall. When operated, a pellet dispenser delivered a 45-mg food pellet (soy-free PJAI, Test Diet, St. Louis, MO) into a pellet trough, which was centrally mounted between the two levers. Located above each lever was a stimulus light. Houselights were centrally mounted on the ceiling. All chambers were equipped with a fan, which supplied ventilation and white noise.

Operant Procedures

Rats were trained to lever press seven days a week, as previously described (McMahon & Cunningham, 2001), with the operant procedure modified for food reinforcement. Initially rats were placed in operant conditioning chambers for 30-90 min, under a fixed ratio (FR)1 schedule, where one lever press on the lever designated by illumination of the light above the lever resulted in delivery of one 45-mg pellet; the second lever was inactive. Rats could receive a maximum of 50 pellets; once response rate (responses per s) during each of three consecutive sessions deviated by no more than +/-20% of the 3-day running average, regardless of rate, the FR was increased in the following increments: FR3, FR6, FR10. Responding under the FR10 schedule continued until response rate was stable, defined as three consecutive sessions that deviated by no more than +/-20% of the 3-day running average. Thereafter, sessions were divided into consecutive, discrete, 20-min cycles. Each cycle began with a 15-min timeout during which the stimulus light was not illuminated and responses on the lever had no programmed consequence. The timeout was followed by a 5-min period during which food pellets were available under the

FR10 schedule. Each session consisted of up to six cycles. Completion of 10 responses resulted in delivery of a food pellet; a maximum of 10 pellets could be delivered per cycle. Rats received vehicle or drug injections within the first min of a given cycle. Drug tests were initiated once responding stabilized, i.e., responding during each of three consecutive sessions deviated by no more than +/-20% of the 3-day running average calculated for all cycles in all sessions.

Hot plate Test

Antinociceptive testing was performed in the hot plate test, as previously described (Ignatowska-Jankowska *et al.,* 2015), but modified for rats. Rats were placed on a heated (52°C) enclosed Hot Plate Analgesia Meter (Columbus Instruments, Columbus, OH) and latency to jump or lick/shake the back paws was determined. If there was no response within 60 s, the rat was removed from the apparatus.

Experimental Design

Immediately following one 20-min cycle in the operant conditioning procedure, rats were placed on the hot plate and tested for their latency to respond to the thermal stimulus. After hot plate testing rats received injections of either vehicle or a drug dose and returned to the operant conditioning chamber for testing in the next cycle. This repeated testing in both the hot plate and operant conditioning chambers occurred a total of six consecutive 20-min cycles per drug test session. As shown in Supplemental Figure 1, exposure to the hot plate did not modify operant response rates, and vice versa, through six consecutive 20-min cycles. After each drug test session, rats received a minimum 48-hour washout period before receiving the next drug. During these washout periods, rats were given daily sessions consisting of 3-6 cycles to lever press for food, without being exposed to the hot plate apparatus.

Drug Combinations

When the mean effect of a drug to reduce food-maintained responding or to produce antinociception was greater than 50%, the ED_{50} values and corresponding 95% confidence limits (CL) were calculated using linear regression, where slopes were allowed to vary, according to Tallarida (2000). Doses for the drug combinations were based upon these calculated ED_{50} values for drugs to reduce schedule-controlled responding for food. Specifically, the log base 2 of a drug's ED_{50}, the ED_{50}, as well as the (1/2) ED_{50}, (1/4) ED_{50}, and (1/8) ED_{50} of a drug to decrease schedule-controlled responding were utilized as experimental doses, which were tested in equipotent combination in both assays.

Data analysis

A within-subject design was used to test dose and dose combinations and the order was nonsystematic. Dose-response curves were determined twice for each assay, once at the beginning of experimental testing and again at the end of the experimental testing. The first determination was used for all experimental calculations, and the second determination was used to examine the development of drug tolerance. Hot plate data were converted to percent maximum possible effect (% MPE) with the following equation: ([(experimental test value - baseline value)/ (maximum test value – baseline value)] x100) and plotted versus log dose values. Response rates were expressed as a percentage of control for each rat, defined as the mean response rate from the previous three non-drug sessions, with each session defined as the individual cycles for that session averaged together. In each experiment, male and female data were analyzed together in all further data analyses. If the mean effect of a drug did not produce a 50% or greater effect, an ED_{50} value was not generated. Potency ratios and 95% CL were calculated as the ratio of ED_{50} values calculated from the dose-response curves, and a potency ratio not including 1within the 95% CL indicated a statistically significant difference in potency.

In the CGP35348 antagonism study on the effects of the combination of baclofen and buspirone on thermal hotplate latency, ED_{50} and potency ratio values could not be calculated, thus an unpaired student's t-test was used to compare potency effects. The theoretical additive ED_{50} value of the combined drugs was calculated from the individual dose-response curves to determine synergistic, additive, or subadditive interactions. The combination was assumed to equal the sum of the effects of each drug. The experimentally derived ED_{50} values (Zmix) from the dose-response curves of the ratios were compared to the predicted additive ED_{50} values (Zadd). If the empirically derived value and the theoretical value did not differ, the interaction was considered additive (Tallarida, 2001; 2006). The degree of effect (i.e., synergy or additivity) in both assays for a drug combination was calculated with the following equation: [(Hot plate Zadd – Hot plate Zmix) / (Rate Zadd – Rate Zmix)], and modified from (Tallarida, 2001). All dose-response data were analyzed using a one-way repeated measures analysis of variance (ANOVA). A Bonferroni comparison was used for post-hoc analysis following a significant ANOVA ($P < 0.05$). Comparisons of initial and rederived dose-response curves were analyzed using a two-way repeated measures ANOVA. The computer program GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA) was used in all statistical analyses. All data are expressed as the mean +/- standard error of the mean (SEM).

Results

Effects of morphine and baclofen on schedule-controlled responding and latency to respond to thermal stimuli

Figure 1 shows the effects of morphine and baclofen on rate of FR10 responding for food, and latency to respond in the hot plate test. Repeated injections of vehicle did not alter rates of responding for food (Supplemental Figure 1A, the range of absolute rate of responding was 0.85-0.96 responses/s) or latency to respond to thermal stimulus (Supplemental Figure 1B, 13.5-17.7 s). Thus, stable baselines were obtained across the six cycles in both schedule-controlled responding ($F7,35 = 1.4$; $P = 0.22$) as well as nociceptive latency ($F7,35 = 2.7$; $P = 0.43$). Morphine and baclofen individually dose-dependently decreased the rate of food-maintained responding (Figure 1A; $F7,21 = 13.7$, $P < 0.001$) and (Figure 1B; $F7,28 = 27.9$, $P < 0.0001$). The ED_{50} values of morphine and baclofen to reduce operant responding were 7.09 (95% CL: 3.60 – 10.58) mg/kg and 3.42 (95% CL: 2.07 – 4.77) mg/kg, respectively. We next assessed the dose-response relationship of equi-effective doses of each drug to reduce schedule-controlled responding, in combination, and as expected, found a leftward shift in the dose-response relationship compared with either compound administered alone (Figure 1A, B). Analysis of the mixture potency (i.e., Zmix) and additive potency (i.e., Zadd) of the combination of morphine and baclofen revealed a synergistic interaction between these drugs. The calculated experimental Zmix (3.73 (2.86 – 4.00) mg/kg) was significantly less than the calculated theoretical Zadd (5.55 (5.28 – 5.82) mg/kg).

Figure 1 also shows the effects of morphine and baclofen, administered separately and together, on the latency to respond in the hot plate test. Both baclofen and morphine dose-dependently increased the latency to respond to thermal stimulus [(Figure 1C; $F7,21 = 49.3$, $P <$

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0.0001), (Figure 1D; $F7,35 = 36.9$, $P < 0.0001$), respectively]. The ED_{50} values of morphine and baclofen to produce thermal antinociception were 16.15 (95% CL: 12.69 – 20.56) mg/kg and 8.75 (95% CL: 6.03 – 12.72) mg/kg, respectively. Operant responding was 2.28-fold more sensitive to the effects of morphine than was thermal nociception (potency ratio: 2.28 (95% CL: 1.94 – 3.52) and 2.56-fold more sensitive to the effects of baclofen than was thermal nociception (potency ratio: 2.56 (95% CL: 2.21 – 2.91). We next assessed the dose-response relationship of equi-effective doses of each drug in combination from Figure 1A and Figure 1B in the hot plate assay and found a leftward shift in the dose-response relationship compared with either compound administered alone (Figure 1C, D). Analysis of the mixture potency and additive potency of the combination of baclofen and morphine revealed a synergistic interaction between these drugs. The calculated experimental Zmix (6.49 (5.57 – 7.41) mg/kg) was significantly less than the calculated theoretical Zadd (13.54 (12.90 – 14.18) mg/kg). The degree of effects from the combination of morphine and baclofen was 3.87-fold higher in the increase of latency to respond to thermal stimulus than the decrease of food-maintained responding.

Effects of morphine and buspirone on schedule-controlled responding and latency to respond to thermal stimuli

Figure 2 shows the effects of morphine and buspirone on rate of FR10 responding for food, and latency to respond in the hot plate test. Buspirone alone dose-dependently decreased the rate of food-maintained responding (Figure 2A; $F7,28 = 25.8$, $P < 0.0001$). The ED_{50} value of buspirone to reduce operant responding was 0.57 (95% CL: 0.31 – 1.07) mg/kg. We next assessed the dose-response relationship of equi-effective doses of buspirone in combination with morphine and found a leftward shift in the dose-response relationship compared with either compound given alone (Figure 2A, B). Analysis of the mixture potency and additive potency

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revealed additivity between these drugs. The calculated experimental Zmix (7.22 (5.23 – 9.21) mg/kg) was not significantly different from the calculated theoretical Zadd (5.70 (5.68 – 5.72) mg/kg).

Figure 2 also shows the effects of morphine and buspirone, administered separately and together, on thermal antinociception. Buspirone dose-dependently increased the latency to respond to thermal stimulus (Figure 2C; $F_{7,28} = 24.0$ P < 0.0001). The ED_{50} value of buspirone to produce thermal antinociception was 2.20 (95% CL: 1.47 – 3.29) mg/kg). Operant responding was 3.86-fold more sensitive to the effects of buspirone than was thermal nociception (potency ratio: 3.86 (95% CL: 3.07 – 4.65)). We next assessed the dose-response relationship of equi-effective doses of morphine and buspirone in combination from Figure 2A and Figure 2B in the hot plate test and found a leftward shift in the dose-response relationship compared with either compound given alone (Figure 2C, D). Analysis of the mixture potency and additive potency revealed a synergistic interaction between these drugs. The calculated experimental Zmix (5.87 (4.35 – 7.39) mg/kg) was significantly less than the calculated theoretical Zadd (14.72 (14.66 – 14.78) mg/kg), and below the line of additivity. The degree of effects from the combination of morphine and buspirone was 5.82-fold higher in the hot plate test.

Effects of baclofen and buspirone on schedule-controlled responding and latency to respond to thermal stimuli

Figure 3 shows the effects of baclofen and buspirone on rate of FR10 responding for food and latency to respond in the hot plate test. We assessed the dose-response relationship of equi-effective doses of baclofen in combination with buspirone and found a leftward shift in the dose-

response relationship compared with buspirone given alone (Figure 3A, B). Analysis of the

mixture potency and additive potency revealed additivity. The calculated experimental Zmix

(2.82 (2.06 – 3.58) mg/kg) was not significantly different from the calculated theoretical Zadd

(4.36 (3.56 – 5.16) mg/kg).

Figure 3 also shows the effects of buspirone and baclofen on thermal antinociception. We

assessed the dose-response relationship of equi-effective doses of baclofen and buspirone in

combination from Figure 3A and Figure 3B in the hot plate test and found a leftward shift in the

dose-response relationship compared with buspirone given alone (Figure 3C, D). Analysis of the

mixture potency and additive potency revealed a synergistic interaction between these drugs. The

calculated experimental Zmix (3.70 (3.00 – 4.40) mg/kg) was significantly less than the

calculated theoretical Zadd (7.38 (6.14 – 8.62) mg/kg). The degree of effects from the

combination of baclofen and buspirone was 2.39-fold higher in the hot plate test.

Effect of 0.032 mg/kg naltrexone on morphine and morphine drug combination-induced

changes in operant responding and latency to respond to thermal stimuli.

By itself, 0.032 mg/kg naltrexone administered during the first cycle, followed by vehicle

in subsequent cycles, did not alter rates of responding for food (Supplemental Figure 1C; $F_{7,37}$ =

3.1; P = 0.07) or latency to respond to thermal stimulus (Supplemental Figure 1D; $F_{7,35}$ = 1.8; P

= 0.19). As indicated by a rightward shift in dose-response functions (Figure 4 panels A and B),

0.032 mg/kg naltrexone antagonized the effects of morphine on operant responding and latency

to respond to thermal stimulus. In the presence of naltrexone, the ED_{50} values of morphine to

reduce operant responding and to produce thermal antinociception were 29.51 (19.23 – 39.79)

and 50.34 (41.93 – 58.78) mg/kg, respectively. A potency ratio analysis confirmed significant

antagonism of morphine by 0.032 mg/kg naltrexone, as evidenced by a 4.16 (95% confidence

limits: 3.76 – 5.34) potency ratio between respective morphine dose-response functions to

decrease operant responding and a 3.12 (95% confidence limits: 2.86 – 3.30) potency ratio

between respective morphine dose-response functions to produce thermal antinociception.

Naltrexone antagonized the combined effects of baclofen and morphine on operant responding

(Figure 4C), as evidenced in a potency ratio of 2.78 (95% confidence limits: 2.35 – 3.28)

between respective dose-response functions, as well as to produce thermal antinociception

(Figure 4D), as evidenced in a potency ratio of 1.69 (95% confidence limits 1.59 – 1.80) between

respective dose-response functions. Naltrexone also antagonized the combined effects of

buspirone and morphine on operant responding (Figure 4C), and to produce thermal

antinociception with a potency ratio analysis confirming an antagonistic effect in both (1.19

(95% confidence limits: 1.02 – 1.38) and 3.52 (95% confidence limits 3.08 – 4.00), respectively).

Effect of 320 mg/kg CGP35348 on baclofen and baclofen drug combination-induced

changes in operant responding and latency to respond to thermal stimuli.

By itself, 320 mg/kg CGP35348 administered during the first cycle, followed by vehicle

in subsequent cycles did not alter rates of responding for food (Supplemental Figure 1E; $F_{6,30} =$

2.8; P = 0.06) or latency to respond to thermal stimulus (Supplemental Figure 1F; $F_{6,30} = 3.3$; P

= 0.08). As indicated by a rightward shift in dose-response functions (Figure 5 panels A and B),

320 mg/kg CGP35348 antagonized the effects of baclofen on operant responding. In the presence

of CGP35348, the ED_{50} values of baclofen to reduce operant responding and to produce thermal

antinociception were 13.62 (6.07 – 30.56) and 17.11 (13.89 – 21.08) mg/kg, respectively. A

potency ratio analysis confirmed significant antagonism of baclofen by 320 mg/kg CGP35348,

as evidenced in a potency ratio of 3.55 (95% confidence limits: 2.46 – 5.14) between respective

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baclofen dose-response functions to decrease operant responding and a potency ratio of 1.96 (95% confidence limits: 1.66 – 2.30) between respective baclofen dose-response functions to produce thermal antinociception. CGP35348 significantly antagonized the combined effects of baclofen and morphine on operant responding (Figure 5C) and latency to respond to thermal stimulus (Figure 5D) as evidenced in potency ratios of (1.52 (95% confidence limits: 1.40 – 1.67) and 1.65 (95% confidence limits 1.39 – 1.96), respectively). However, CGP35348 did not significantly antagonize the effects of combined baclofen and buspirone on operant responding (Figure 5E) with a potency ratio analysis confirming no significant change in baclofen's effect (1.00 (95% confidence limits: 0.60 – 1.40). Thermal antinociception from the highest dose combination of 6.83 mg/kg baclofen and 1.14 mg/kg buspirone was significantly antagonized by 320 mg/kg CGP35348, as indicated by the results of a t-test ($P < 0.05$).

Development of tolerance to baclofen, morphine and buspirone on schedule-controlled responding and thermal nociceptive latency.

After all studies were completed, dose-response curves of baclofen, morphine, and buspirone were redetermined for each drug to decrease schedule-controlled responding and to produce thermal antinociception (Supplemental Figure 2). Baclofen underwent significant tolerance in schedule-controlled responding [significant interaction between treatment and time: ($F_{4,48} = 2.64$; $P < 0.05$)] but did not undergo significant tolerance in the hot plate test [interaction between treatment and time: ($F_{4,48} = 2.64$; $P = 0.78$)], (Supplemental Figure 2A and B). Conversely, morphine did not undergo significant tolerance in schedule-controlled responding [interaction between treatment and time: ($F_{3,36} = 2.02$; $P = 0.13$)], but did undergo significant tolerance in the hot plate test [significant interaction between treatment and time: ($F_{3,36} = 3.80$; $P < 0.05$)], (Supplemental Figure 2C and D). Meanwhile, buspirone underwent significant

sensitization in schedule-controlled responding [significant interaction between treatment and time: ($F_{4,48} = 7.65$; $P < 0.0001$)], but underwent significant tolerance in the hot plate test [significant interaction between treatment and time: ($F_{4,48} = 3.55$; $P < 0.05$)], (Supplemental Figure 2E and F).

Discussion

Here we show the potency for morphine, baclofen, and buspirone to decrease schedule-controlled responding for food is higher than that needed to produce thermal antinociception. The effects of morphine plus baclofen to decrease schedule-controlled responding for food and to produce thermal antinociception were synergistic. The effects of morphine plus buspirone and baclofen plus buspirone on schedule-controlled responding for food were additive, and the effects of the two combinations to increase the latency to respond to thermal stimulus were synergistic. As dose combination experiments were completed in a randomized order, it is difficult to determine the overall contribution to the development of tolerance or sensitivity, specifically regarding the rate-decreasing effects of buspirone, of drug effects on the observed experimental outcomes. These findings suggest that buspirone may produce synergistic acute antinociception when combined with morphine, with only additive or less-enhanced effects on other behaviors. Further, these findings over the combination of baclofen plus buspirone may represent novel $GABA_B$ and $5-HT_{1A}$ receptor interactions, which might be further optimized to yield novel therapeutics for the treatment of pain with a lessened side-effect profile.

A dose of naltrexone that antagonized morphine's effects in schedule-controlled responding and thermal antinociception also antagonized both these behavioral effects when morphine was combined with baclofen, and separately, when morphine was combined with buspirone. Thus, opioid receptors were involved in the effects produced when morphine was combined with either baclofen or buspirone to decrease in schedule-controlled responding, as well as to produce thermal antinociception. A dose of CGP34358 that antagonized baclofen's effects in schedule-controlled responding and thermal antinociception also antagonized both these behavioral effects when baclofen was combined with morphine. However, when compared

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to the effects of CGP34358, naltrexone produced a greater rightward shift of the baclofen plus

morphine dose-response curve in both behavioral measures. Thus, it is likely that opioid

receptors played a bigger role in the effects of the baclofen plus morphine drug combination than

$GABA_B$ receptors. Interestingly, this same dose of CGP34358 failed to antagonize the effects of

baclofen plus buspirone on schedule-controlled responding, and only antagonized the highest

studied dose combination of baclofen and buspirone in the hot plate test. From this, it is likely

that the actions of buspirone at multiple receptors (i.e., buspirone can as a serotonin $(5\text{-}HT)_{1A}$

receptor agonist, or a dopamine D_2, D_3, and D_4 antagonist) accounts for the observed

pharmacological effects of the combination of baclofen plus buspirone.

To the authors' knowledge, the examination of the combination of baclofen and

buspirone on the effects of thermal antinociception or scheduled-controlled response behavior

has not been reported in the literature. Here we show that the combination of baclofen and

buspirone synergistically increased latency to respond to thermal stimulus, and only additively

disrupted schedule-controlled responding.

There are several lines of evidence that support the therapeutic combination of baclofen

with morphine. There are numerous studies that suggest that $GABA_B$ agonists possess relatively

low abuse potential and may in fact be useful as therapeutics to treat drug abuse. Previous studies

have shown that baclofen decreases the reinforcing effects of opioids in rat self-administration

procedures (Xi & Stein,1999; Ramshini *et al.,* 2013). Treatment with a $GABA_B$ inhibitor

phaclofen seemingly increased morphine self-administration (Ramshini *et al.,* 2013). Similarly,

baclofen has been found to abolish morphine preference in the place conditioning assay in mice

(Meng *et al.,* 2014). These anti-rewarding effects of baclofen are attributed to the actions of

$GABA_B$ receptor stimulation to inhibit dopamine release. Specifically, direct administration of

baclofen into the ventral tegmental area has been found to inhibit the local release of opioid-induced dopamine (Klitenick *et al.,* 1992; Xi & Stein, 1999). Systemic administration of baclofen has been found to inhibit the release of opioid-induced dopamine in the nucleus accumbens (Fadda *et al.,* 2003; Fu *et al.,* 2012). This baclofen-induced decrease of dopamine release in the nucleus accumbens has also been found in correlation with repeated administration of baclofen with morphine, where baclofen and morphine co-administration prevented the development of tolerance to the effects of morphine on locomotor activity in rats (Fu *et al.,* 2012; Topkara *et al.,* 2017). In addition to directly modifying the rewarding properties of opioids, baclofen has also been found to reduce behavioral signs of naloxone-induced morphine withdrawal in mice (Pedron *et al.,* 2016) and rats (Topkara *et al.,* 2017). Additional studies have shown that baclofen can decrease the rewarding effects of other commonly abused drugs such as nicotine, cocaine, and methamphetamine (Ranaldi & Poeggel 2002; Fadda *et al.,* 2003). It was previously found that baclofen decreased morphine-induced nausea and vomiting in ferrets (Suzuki *et al.,* 2005). In a retrospective clinical study, multiple sclerosis patients that had been on intrathecal (peri-spinal) baclofen for muscle spasticity but experienced uncontrolled pathological pain responded well with the addition of intrathecal morphine for pain, with limited side-effects reported (Sadiq & Poopatana, 2007). However, our studies suggest that baclofen may not be suitable in combination with opioids for acute pain management, due to synergistic drug-induced disruptions in schedule-controlled responding.

Although limited, the existing body of literature also suggests a favorable profile of the combination of buspirone with opioids for pain management. In rats, buspirone has been found to decrease the rewarding effects of morphine in the conditioned place preference assay, prevent the development of tolerance to morphine's locomotor effects, and enhance the effects of

morphine in the hot plate assay (Haleem & Nawaz, 2017). In non-human primate studies, buspirone diminished the ability of animals to detect morphine in a drug discrimination study and enhanced the effects of morphine to produce thermal antinociception in the tail-flick test (Li *et al.,* 2011). Although buspirone has effects at multiple receptors, it can act as a 5-HT$_{1A}$ receptor agonist. It has been well established that less sub-type selective 5-HT receptor agonists produce thermal antinociception and enhance the antinociceptive effects of opioids in rodents and non-human primates (Larson & Takemori, 1977; Banks *et al.,* 2010). A small prospective German clinical study of 12 females, 20-30 years of age, found that a single dose of oral buspirone did not alter the thermal nociceptive responses of healthy volunteer subjects when compared to a single intravenous morphine dose in the same testing paradigm (Pavlakovic *et al.,* 2009). Although the results of this study reveal a negative finding, there are a considerable number of experimental factors (i.e., relatively small homogenous experimental cohort, differing routes of drug administration, single drug doses) that limit the interpretation of the results. Further, as the experimental cohort in this study was comprised solely of young, pre-menopausal women, estrus stage was not reported. Of importance, estrus cycle stage has been reported to influence acute pain perception in healthy women (Hellstrom & Lundberg, 2000). Thus, additional clinical studies comprised of both men and women are needed to identify if buspirone alone can produce antinociception in humans, and to examine, as reported in the current and other preclinical studies, whether buspirone in combination with opioids produce either additive or synergistic effects in the production of antinociception.

One caveat to our current study is that we only examined 1:1 combinations of drugs, based upon ED$_{50}$ values. Differential dose ratio combinations (i.e., 1:3, 3:1 ratios) are valuable pharmacological assessments for drug combination optimization. Specifically, a bigger dose of a

drug, based upon dose equivalence, allows for the ability to select preferable pharmacological interactions, which is useful when maximizing relative therapeutic vs. side-effect profiles (Foucquier & Guedj, 2015). Here we only assessed decreases in schedule-controlled responding as a potential untoward side-effect of drugs alone and in combination. Further studies, outside of the scope of the current study, are needed to examine other potential side-effects of these drug combinations, specifically sedation and respiratory depression, in comparison to therapeutic pain outcomes. Another important consideration of this data is that here we examine the effects of baclofen, buspirone, morphine, and respective combinations of these drugs in a model of acute nociception. The hot plate test has relatively good predictive validity for antinociceptive compounds, especially opioids (Morgan & Christie, 2011; Taber, 1973). Importantly, rats do not exhibit grimace behaviors during acute painful stimuli such as the hot plate test (Langford *et al.*, 2010). Thus, measuring non-reflexive acute nociceptive behaviors would require significant pain stimulation. However, cellular processes underlying behaviors associated with pathological pain are different than those of acute nociception (De Leo *et al.,* 2006; Milligan & Watkins, 2009; Wilkerson & Milligan, 2011) and thus results shown here for acute reflexive nociceptive inhibition may not generalize to behavioral effects observed in preclinical models of pathological pain. Further studies are needed to examine whether these drugs in multiple combinations (i.e., 1:1, 1:3. 3:1) produce reversal of pain-evoked and pain-depressed behaviors (Negus *et al.,* 2010; Wilkerson *et al.,* 2018) in preclinical models of pathological pain.

Here we show that effects of the combination of morphine plus baclofen to decrease schedule-controlled responding for food and to produce thermal antinociception were synergistic, with a 3.87-fold higher degree of effect for an increased effect in thermal latency than a decreased effect in schedule-controlled responding. Meanwhile, effects of the combination

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of morphine plus buspirone and baclofen plus buspirone on schedule-controlled responding for

food were additive, and the effects of the two combinations to increase latency to respond to

thermal stimulus were synergistic, with a 5.82- and 2.39-fold higher degree of effect in the hot

plate assay, respectively. Synergistic antinociceptive effects, in conjunction with additive

behaviorally disruptive effects, highlights the utility of opioid and non-opioid drug combinations

as effective pain therapeutics.

Participated in research design: Wilkerson, McMahon

Conducted experiments: Felix, Restrepo

Contributed new reagents or analytic tools: Ansari, Coop

Performed data analysis: Wilkerson, McMahon

Wrote or contributed to the writing of the manuscript: Wilkerson, McMahon

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Footnotes

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Figure Legends

Figure 1. Systemic baclofen and morphine decrease schedule-controlled responding for food and produce thermal antinociception. (A) Baclofen alone and in combination with equi-effective doses of morphine decreases schedule-controlled behavior. (B) Morphine alone and in combination with equi-effective doses of baclofen decreases schedule-controlled behavior. (C) Baclofen alone and in combination with morphine increases latency to respond to thermal stimulus. (D) Morphine alone and in combination with baclofen increases latency to respond ti thermal stimulus. The ordinate in (A), (C) depicts the rate of responding calculated as a percentage of control, and in (B), (D) the maximum percent effect (MPE) as a percentage of latency to respond to thermal stimulus. The abscissa depicts the dose of drug administered. *P < 0.05, **P < 0.01, *** P < 0.001, **** P < 0.0001 vs. vehicle. Data reflect mean ± SEM, n = 8 rats/group.

Figure 2. Systemic buspirone and morphine decrease schedule-controlled responding for food and produce thermal antinociception. (A) Buspirone alone and in combination with equi-effective doses of morphine decreases schedule-controlled behavior in a dose-related manner. (B) Morphine alone and in combination with equi-effective doses of buspirone decreases schedule-controlled behavior. (C) Buspirone alone and in combination with morphine increases latency to respond to thermal stimulus. (D) Morphine alone and in combination with buspirone increases latency to respond to thermal stimulus. The ordinate in (A), (C) depicts the rate of responding calculated as a percentage of control, and in (B), (D) the maximum percent effect (MPE) as a percentage of latency to respond to thermal stimulus. The abscissa depicts the dose of drug administered. *P < 0.05, **P < 0.01, *** P < 0.001, **** P < 0.0001 vs. vehicle.

Morphine dose-response curves are the same as those shown in Figure 1. Data reflect mean ±

SEM, n = 8 rats/group.

Figure 3. Systemic baclofen and buspirone decrease schedule-controlled responding for food and

produce thermal antinociception. (A) Baclofen alone and in combination with equi-effective

doses of buspirone decrease schedule-controlled behavior. (B) Buspirone alone and in

combination with equi-effective doses of baclofen decrease schedule-controlled behavior. (C)

Baclofen alone and in combination with buspirone increased latency to respond to thermal

stimulus in a dose-related manner. (D) Buspirone alone and in combination with baclofen

increased latency to respond to thermal stimulus. The ordinate in (A), (C) depicts the rate of

responding calculated as a percentage of control, and in (B), (D) the maximum percent effect

(MPE) as a percentage of latency to respond to thermal stimulus. The abscissa depicts the dose

of drug administered. $*P < 0.05$, $**P < 0.01$, $*** P < 0.001$, $**** P < 0.0001$ vs. vehicle.

Baclofen and buspirone dose-response curves are the same as those shown in Figure 1 and

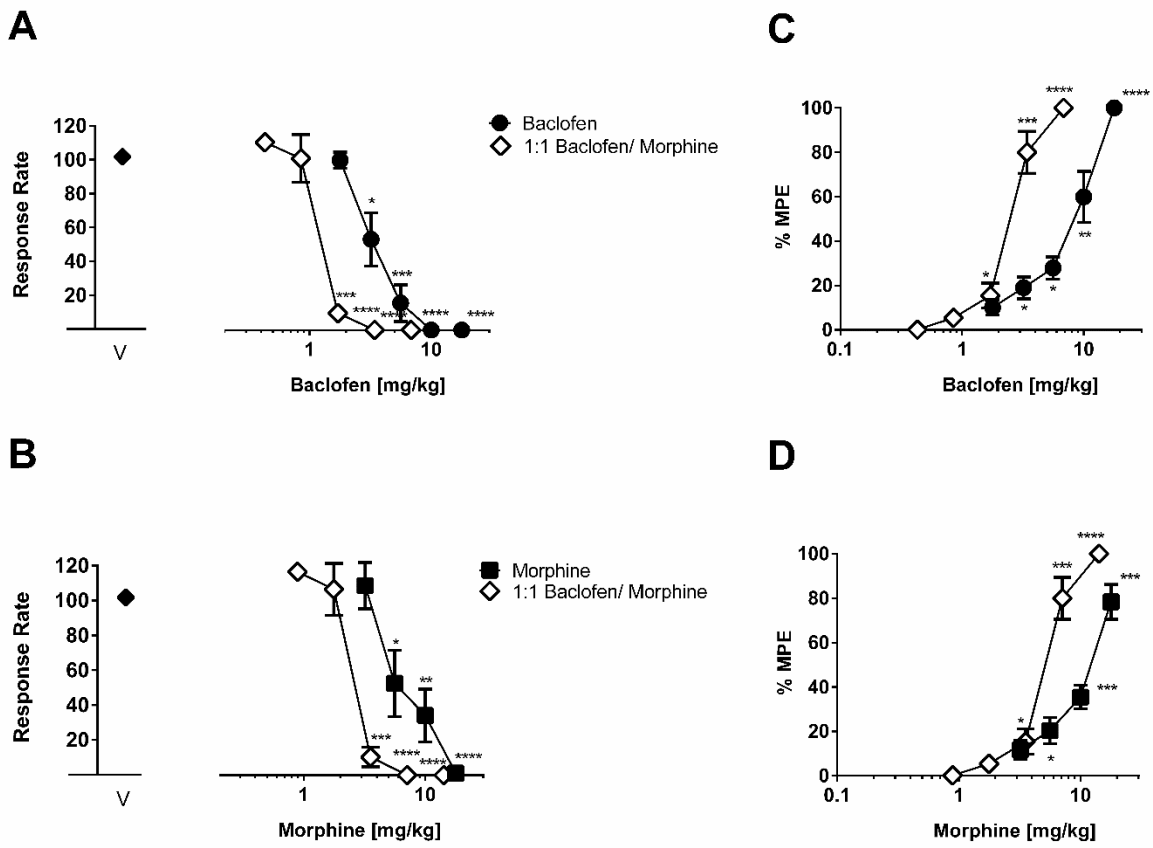Figure 2, respectively. Data reflect mean ± SEM, n = 8 rats/group.

Figure 4. Effects of pretreatment with naltrexone on drug-induced changes in food-maintained

responding and thermal antinociception. Abscissae: drug dose (mg/kg, I.P.), log scale; ordinates:

(A), (C), and (E): Percent control rates of food-maintained responding. (B), (D), and (F): Percent

maximum possible effect in the hot plate test. (A), (B), The dose dependent effects of morphine,

(C), (D), equi-effective dose combinations of baclofen and morphine and (E), (F), equi-effective

dose combinations of buspirone and morphine with pretreatment with naltrexone. $*P < 0.05$, $**P$

< 0.01, $*** P < 0.001$, $**** P < 0.0001$ vs. vehicle or naltrexone, respectively according to

pretreatment. The morphine dose-response curves are the same as those shown in Figure 1. Data

reflect mean ± SEM, n = 8 rats/group.

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Figure 5. Effects of pretreatment with CGP35348 on drug-induced changes in food-maintained responding and thermal antinociception. Abscissae: drug dose (mg/kg, I.P.), log scale; ordinates: (A), (C), and (E): Percent control rates of food-maintained responding. (B), (D), and (F): Percent maximum possible effect in the hot plate test. (A), (B), The dose dependent effects of buspirone, (C), (D), equi-effective dose combinations of baclofen and morphine and (E), (F), equi-effective dose combinations of buspirone and baclofen with 320 mg/kg CGP35348 pretreatment. *P < 0.05, **P < 0.01, *** P < 0.001, **** P < 0.0001 vs. vehicle or CGP35348, respectively according to pretreatment. The baclofen dose-response curves are the same as those shown in Figure 1. Data reflect mean ± SEM, n = 7-8 rats/group (one rat was lost to attrition).
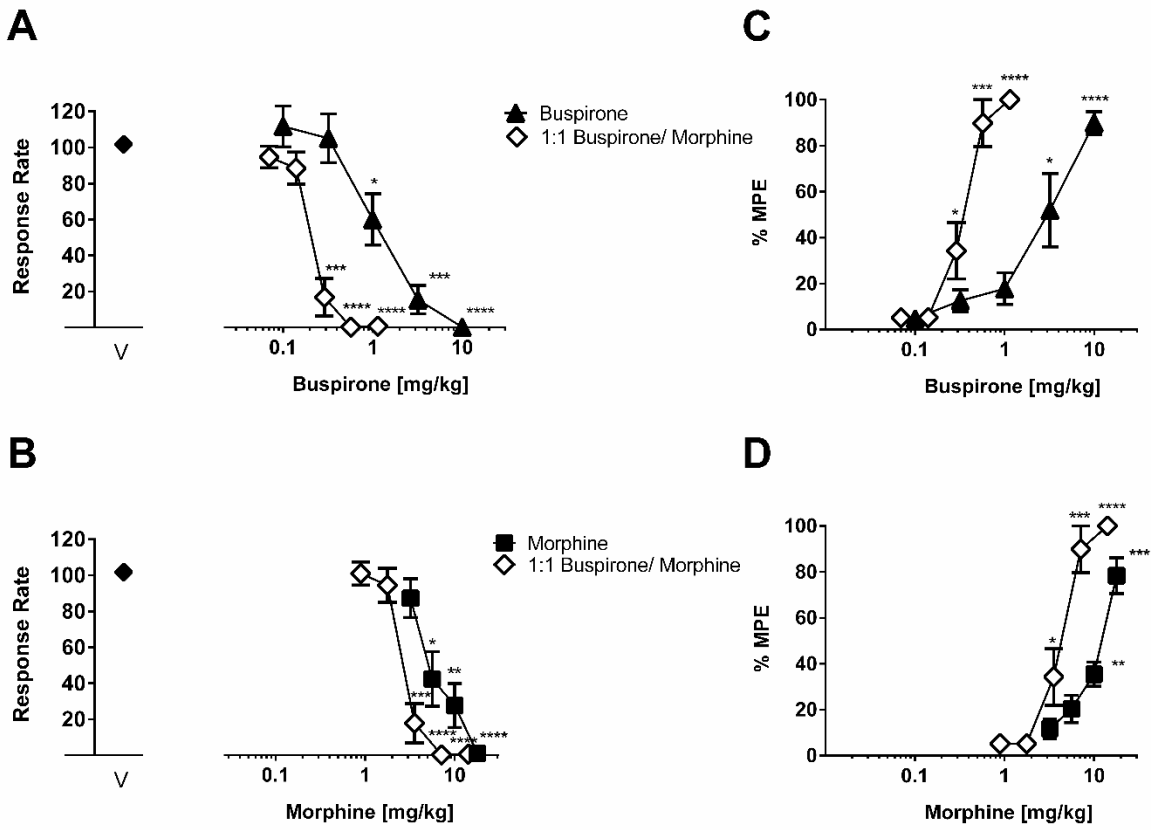
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Figure 1

Figure 2

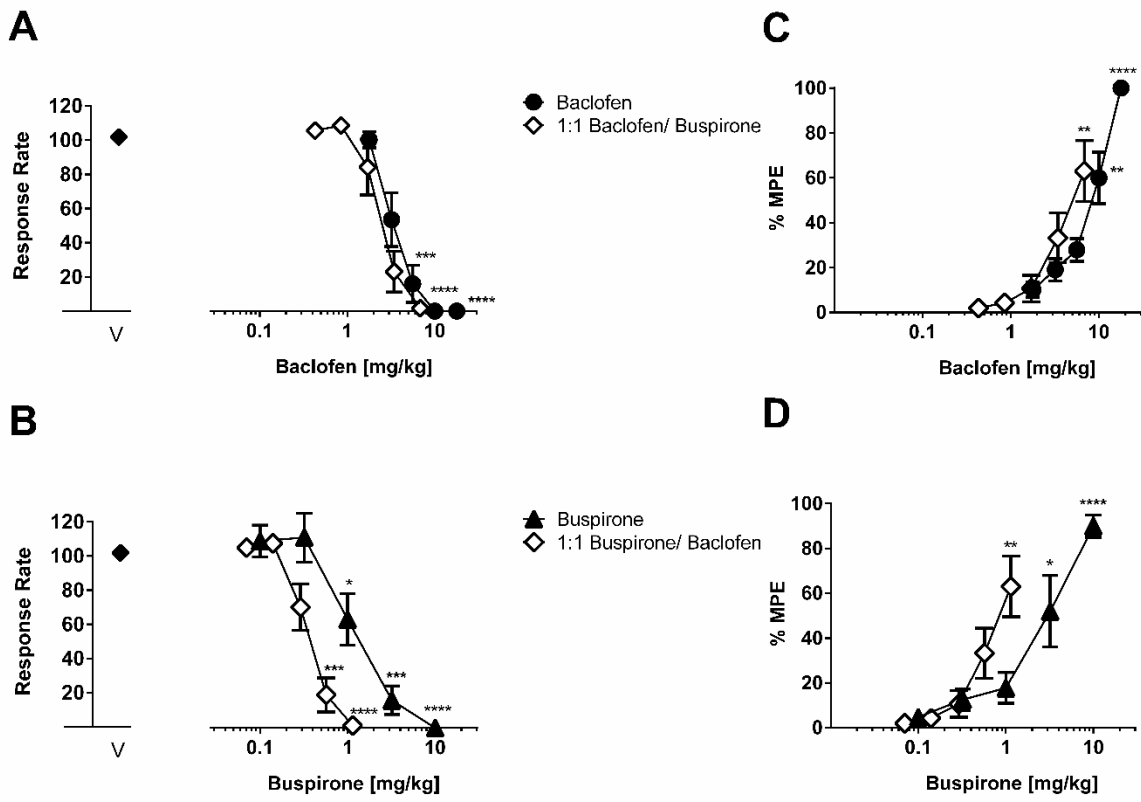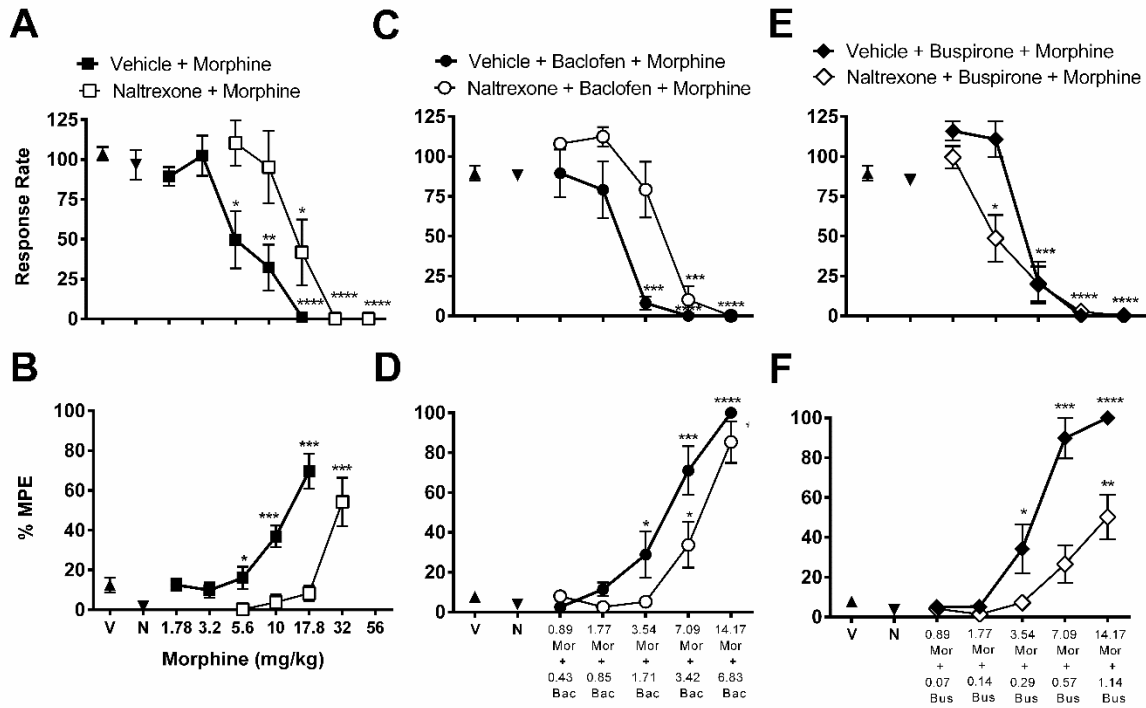Figure 3

Figure 4

Figure 5