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Title Page

Pharmacological Comparison of Mitragynine and 7-Hydroxymitragynine: *In Vitro* Affinity and Efficacy for Mu-Opioid Receptor and Opioid-Like Behavioral Effects in Rats

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Running Title Page

Running title: Pharmacology of Mitragynine at Mu-Opioid Receptors

ABSTRACT

Relationships between μ-opioid receptor (MOR) efficacy and effects of mitragynine and 7-hydroxymitragynine are not fully established. We assessed *in vitro* binding affinity and efficacy, and discriminative-stimulus effects together with antinociception in rats. The binding affinities of mitragynine and 7-hydroxymitragynine at MOR (K_i values 77.9 and 709 nM, respectively) were higher than their binding affinities at κ- (KOR) or δ-opioid receptors (DOR). [^{35}S]GTPγS stimulation at MOR demonstrated that mitragynine was an antagonist, whereas 7-hydroxymitragynine was a partial agonist (Emax=41.3%). In separate groups of rats discriminating either morphine (3.2 mg/kg) or mitragynine (32 mg/kg), mitragynine produced a maximum of 72.3% morphine-lever responding, and morphine produced a maximum of 65.4% mitragynine-lever responding. Other MOR agonists produced high percentages of drug-lever responding in the morphine and mitragynine discrimination assays: 7-hydroxymitragynine (99.7% and 98.1%, respectively), fentanyl (99.7% and 80.1%, respectively), buprenorphine (99.8% and 79.4%, respectively), and nalbuphine (99.4% and 98.3%, respectively). In the morphine and mitragynine discrimination assays, the KOR agonist U69,593 produced maximums of 72.3% and 22.3%, respectively, and the DOR agonist SNC 80 produced maximums of 34.3% and 23.0%, respectively. 7-Hydroxymitragynine produced antinociception; mitragynine did not. Naltrexone antagonized all of the effects of morphine and 7-hydroxymitragynine; naltrexone antagonized the discriminative stimulus effects of mitragynine but not its rate-decreasing effects. Mitragynine increased the potency of the morphine discrimination yet decreased morphine antinociception. Here we illustrate striking differences in MOR efficacy, with mitragynine having less than 7-hydroxymitragynine.

SIGNIFICANCE STATEMENT

At human MOR *in vitro*, mitragynine has low affinity and is an antagonist, whereas 7-hydroxymitragynine has 9-fold higher affinity than mitragynine and is a MOR partial agonist. In rats, intraperitoneal mitragynine exhibits a complex pharmacology including MOR agonism; 7-hydroxymitragynine has higher MOR potency and efficacy than mitragynine. These results are consistent with 7-hydroxymitragynine being a highly selective MOR agonist, and with mitragynine having a complex pharmacology that combines low efficacy MOR agonism with activity at non-opioid receptors.

INTRODUCTION

Opioid overdose, a leading cause of death for people under age 50 in the Unites States (US), has resulted in decreased life expectancy (Crimmins and Zhang, 2019; Melton and Melton, 2019). Current Food and Drug Administration (FDA)-approved medications to treat opioid use disorder include methadone, buprenorphine and naltrexone. However, 40%—60% of patients relapse while being maintained on the currently approved treatments (NIDA, 2018). Thus, there is a need for more effective medications to achieve higher levels of abstinence than those with the FDA-approved medications.

Kratom (*Mitragyna speciosa*), a plant native to Southeast Asia, has been used in Malaysia and Thailand to mitigate opioid withdrawal symptoms (Vicknasingam et al., 2010). Kratom use has increased significantly in the West where kratom products are used for the treatment of pain and opioid dependence, as well as for recreational purposes. More than 40 alkaloids have been identified in kratom leaves, with mitragynine being the most abundant and accounting for 40%—60% of the total alkaloid content (Dargan and Wood, 2013; Hassan et al., 2013). Three diastereomers of mitragynine present in kratom (speciogynine, speciocilliatine, and mitracilliatine) account for an additional 5%—10% of the total alkaloid content (Dargan and Wood, 2013; Hassan et al., 2013; Gogineni et al., 2014). The successful isolation of mitragynine from its diastereomers and related alkaloids has been an overlooked pitfall in the separation process, due to the similar physicochemical properties of kratom alkaloids which might compromise the purity of the individual alkaloids (Sharma et al., 2019).

Mitragynine has received much attention due to its μ-opioid receptor (MOR) pharmacology. For example, mitragynine was a partial agonist at mouse MORs but inactive up to 1.0 μM at δ- or κ-opioid

receptors (DOR and KOR, respectively) using a guanosine 5'-*O*-[γ-thio]triphosphate (GTPγS) functional assay (Varadi et al., 2016). In mice, mitragynine was 2.6-fold less potent than codeine, a prodrug of the MOR agonist morphine, at producing antinociception using a hotplate test (Macko et al., 1972). The antinociceptive effects of mitragynine were blocked by the non-selective opioid antagonist naloxone in mice and were absent in mice lacking MORs and with δ- and κ-opioid receptors intact (Matsumoto et al., 1996b; Kruegel et al., 2019). Results from additional *ex vivo* and *in vivo* studies indicate that the activity of mitragynine may extend beyond MOR. For example, the discriminative-stimulus effects of mitragynine in rats were not blocked by naloxone (Harun et al., 2015), while the inhibitory effects of mitragynine on the contraction elicited by electrical stimulation in the guinea-pig ileum were blocked by naloxone (Watanabe et al., 1997), naltrindole (DOR antagonist), and norbinaltorphimine (KOR antagonist), but *not* by naloxonazine (MOR antagonist) (Shamima et al., 2012). Several studies have reported that mitragynine is a G-protein biased agonist at human MORs (hMORs; Kruegel et al., 2016) and is not self-administered at rates above vehicle when it is substituted for methamphetamine or morphine in rats (Yue et al., 2018; Hemby et al., 2019). Collectively, these findings suggest that mitragynine may be unique among other opioid agonists, whereas 7-hydroxymitragynine appears to be a more consistent opioid agonist (Varadi et al., 2016). However, it is not known to what extent the behavioral effects of mitragynine and 7-hydroxymitragynine reflect differences in MOR efficacy (i.e., intrinsic activity), evidenced by differences in maximum effects and antagonism of higher efficacy MOR agonists.

The present study assessed the *in vitro* and *in vivo* opioid receptor pharmacology of mitragynine extracted from a kratom product at greater than 98% purity (Hiranita et al., 2019), and 7-hydroxymitragynine synthesized from mitragynine as previously described (Obeng et al., 2020). Binding affinity was assessed through displacement of radioligand binding at human opioid receptor subtypes, and MOR efficacy was assessed with a [^{35}S]GTPγS assay. Whereas prior studies have used male subjects to evaluate the *in vivo* pharmacology of kratom alkaloids, both males and females were studied here to address potential sex differences in opioid pharmacology as previously described (e.g. Craft et al., 1996). Female and male rats were trained to discriminate either mitragynine or morphine from vehicle; these discrimination assays were

used to assess substitution profiles with various opioid agonists [high- (fentanyl) and low-efficacy (buprenorphine, and nalbuphine) μ-, κ- (U69,593), and δ-opioid- receptor (SNC 80) agonists]. A hotplate assay was further employed to compare antinociceptive effects. Reversibility of the behavioral effects of mitragynine and 7-hydroxymitragynine was assessed with naltrexone.

MATERIALS AND METHODS

Compounds. The following salt and enantiomeric forms of the drugs were used: [^{3}H][D-Ala^2, D-Leu^5]-Enkephalin ([^{3}H]DADLE) (PerkinElmer, Boston, MA), [^{3}H][D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin ([^{3}H]DAMGO) (PerkinElmer), [^{3}H]U69,593 (PerkinElmer), buprenorphine hydrochloride (National Institute on Drug Abuse, Drug Supply Program, Rockville, MD), DAMGO (Tocris Bioscience, Bristol, UK), DADLE (Tocris Bioscience), fentanyl hydrochloride (National Institute on Drug Abuse), (-)-mitragynine hydrochloride [extracted as described in Hiranita *et al*. (2019)] and (-)-7-hydroxymitragynine [semi-synthesized from mitragynine as in Obeng *et al*. (2020)], (-)-morphine sulfate pentahydrate (National Institute on Drug Abuse), (-)-naltrexone hydrochloride (Sigma-Aldrich Co., St. Louis, MO), nalbuphine (Sigma-Aldrich Co.), U69,593 (Sigma-Aldrich Co.), and SNC 80 (Tocris Bioscience). Dose/concentration is expressed as the weight of the salt form listed above, or as base if no salt form is noted. For *in vitro* studies, compounds were dissolved in dimethyl sulfoxide (Sigma-Aldrich Co.) to form stock concentrations of 10 mM. For behavioral studies, a vehicle consisting of sterile water containing 5% Tween 80 (polyoxyethylenesorbitanmonooleate, Sigma-Aldrich Co.) and 5% propylene glycol (Sigma-Aldrich Co.) was used. Each solution was filtered with a 0.2-μm pore size syringe filter (Millex-LG, 0.20 μm, SLLG025SS, Cole-Parmer, Vernon Hills, IL), and compounds and vehicle were administered intraperitoneally (i.p.) in a volume of 1.0 mL/kg of body weight except mitragynine, 7-hydroxymitragynine and SNC 80, which were prepared in volumes of 1.0—10 mL/kg due to limited solubility. Mitragynine was tested up to 56 mg/kg; a dose of 100 mg/kg of mitragynine was lethal. Mitragynine and naltrexone were administered 30 min prior to sessions; other compounds were administered 15 min prior to sessions. The dose and pretreatment time ranges of the compounds studied were based on our preliminary data and literature (Hiranita et al., 2014; Harun et al., 2015; Tanda et al., 2016; Obeng et al., 2020).

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Receptor Binding Assay. [^{3}H]DADLE, [^{3}H]U69,593 and [^{3}H]DAMGO were used to label the δ-, κ- and μ-opioid receptors, respectively (Barrett and Vaught, 1983; Lahti et al., 1985; Onogi et al., 1995). The K_d and B_{max} values for the radioligands were determined using a saturation assay (Table 1). Monoclonal human opioid receptors were stably expressed in Chinese hamster ovary (CHO) cell lines for δ- (provided by Dr. Stephen J. Cutler, University of South Carolina) and μ-opioid receptors (PerkinElmer) and in human embryonic kidney (HEK) cells for KOR (Dr. Stephen J. Cutler, University of South Carolina). The Bradford protein assay was utilized to determine and adjust the concentration of protein required for the assay (Tal et al., 1985). Ten μg of each membrane protein was separately incubated with the corresponding radioligand in the presence of different concentrations of test compounds in TME buffer (50 mM Tris (Sigma-Aldrich), 3 mM $MgCl_2$ (Sigma-Aldrich), and 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Sigma-Aldrich), pH 7.7) for 60 min at room temperature. The bound radioligand was separated by filtration using the Connectorate filtermat harvester for 96-well microplates (Dietikon, Switzerland) and counted for radioactivity using a Hidex sense beta microplate reader (Hidex, Turku, Finland). Specific binding at δ-, κ- and μ-opioid receptors was determined as the difference in binding obtained in the absence and presence of 10 μM SNC 80, 10 μM U69,593 and 10 μM naltrexone, respectively.

[^{35}S]GTPγS Functional Assay. MOR efficacy was assessed with the [^{35}S]GTPγS functional assay (Harrison and Traynor, 2003). Twenty μg of hMOR-CHO membrane protein was incubated with 10 μM guanosine diphosphate (GDP), 0.1 nM [^{35}S]GTPγS and varying concentrations of the compound under investigation for 1.5 hr at 25°C. In the test for antagonism, a 10-fold higher concentration of the K_i values (Smith et al., 2020) was used for 7-hydroxymitragynine (779 nM), buprenorphine (9.03 nM), mitragynine (7.06 μM), nalbuphine (110 nM), and naltrexone (18.4 nM); these were incubated with increasing concentrations of DAMGO to surmount antagonism. Nonspecific binding was determined with 40 μM unlabeled GTPγS. TME buffer (50 mM Tris-HCl, 9.0 mM $MgCl_2$, 0.2 mM EGTA, pH 7.4) with 150 mM NaCl and 0.14% bovine serum albumin (BSA) was used to increase agonist-stimulated binding; the final volume in each well was 300 μL. Ten μM of DAMGO was included in the assay as the maximum effective concentration at MOR. After the incubation, the bound radioactive ligand was separated from the free radioligand by filtration through a GF/B

glass fiber filter paper and rinsed three times with ice-cold wash buffer (50 mM Tris-HCl, pH 7.2) using the Connectorate harvester. Radioactivity was measured with the Hidex sense beta microplate reader scintillation counter. All assays were determined in triplicate and repeated at least three times.

Animals. Adult female and male Sprague Dawley rats (Taconics, Germantown, NY, N=8 per sex), weighing approximately 250 and 300 g upon arrival, respectively, were singly acclimated for at least three days to a temperature- (21.9°C ± 1.9°C) and humidity-controlled (53% ± 14%) vivarium with a 12-h light/dark cycle (lights on at 0700 h). Food (2918 Teklad global 18% protein rodent diets, Envigo, Frenchtown, NJ) and reverse osmosis water were available in the home cage. After the acclimation period, individual body weights were maintained at 90% of the free-feeding weight, as determined by normative growth curves, by adjusting daily amounts of food (Dustless Precision Pellets Grain-Based Rodent Diet, Bio-Serv, Frenchtown, NJ) that were provided 30 min following daily experimental sessions, in addition to 45-mg sucrose pellets (Dustless Precision Pellets® 45 mg, Sucrose, Bio-Serv) available during experimental sessions. Behavioral protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and were written in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All experiments were conducted in the light cycle at the same time each day seven days per week. The body weight of each subject was measured daily before experiments.

Apparatus. For antinociception testing, a clear acrylic cage surrounded the Hot Plate Analgesia Meter (1440 Analgesia Hot Plate with RS-232 Port and Software, Columbus Instruments, Columbus, OH) to confine the animal during experimental sessions. Temperature on the plate surface was stably maintained at 52°C ± 0.1°C for at least 30 min prior to each use. For drug discrimination testing, 16 operant-conditioning chambers (Model ENV-008; Med Associates Inc., Fairfax, VT) were each enclosed within a sound-attenuating cubicle equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two retractable, 5-cm-long response levers, 5 cm from the midline and 9 cm above the grid floor. A downward displacement of each lever with a force approximating 0.20 N defined a

response. Two amber light-emitting diodes (LEDs) were positioned above each lever (one LED per lever). Sucrose pellets (Dustless Precision Pellets® 45 mg, Sucrose, Bio-Serv) were delivered via a dispenser (Model ENV-203-20; Med Associates Inc.) to a receptacle mounted on the midline of the front wall between the two levers and 2 cm above the floor. A house light was mounted on the wall opposite to the levers. Each operant conditioning chamber was connected to a Dell desktop computer (Intel® Core™ i7-7700 3.60 GHz processor, 16.0 GB of RAM, Microsoft® Windows 10) through an interface (MED-SYST-8, Med Associates Inc.). Med-PC software version V (Med Associates Inc.) controlled experimental events and provided a record of responses. Each rat was assigned to an operant conditioning chamber and that assignment remained the same throughout the study.

Antinociception. Each rat was manually placed on the heated plate; baseline response latency was determined manually using a stopwatch (Martin Stopwatch, Martin Sports, Carlstadt, NJ) by trained and experimentally blinded raters. Latency was defined as the interval that elapsed between placing the rat onto the heated surface and observation of one of the following responses: jumping, paw lick, and paw shake; the maximum latency was 60 sec. Immediately after a response or 60 sec, whichever occurred first, the animal was removed from the apparatus. Following the measurement of hotplate baseline latency, each subject received an i.p. injection and was returned to their home cage. Hotplate response latency was measured a second time immediately following the drug discrimination test session.

Drug Discrimination Training. Each daily experimental session commenced by placing an experimental subject in the operant conditioning chamber; the initial session duration was 120 min. Both retractable levers were presented and the LED above each lever was illuminated. Each downward deflection of the lever turned off the LEDs and activated the pellet dispenser for 0.1 sec [fixed-ratio (FR) 1 schedule] followed by a 0.1-sec timeout, during which the LEDs were turned off, the house light was illuminated, and responding had no scheduled consequences; the retractable levers remained presented during the timeout. The correct lever (left versus right) was alternated daily, and the ratio value was systematically increased each session. After 50 reinforcers per session were delivered within 20 min for two consecutive sessions under the FR10 schedule of reinforcement, drug discrimination training was initiated.

Experimental subjects were divided into two groups: one trained to discriminate morphine (3.2 mg/kg, i.p., administered 15 min prior to sessions) and a second group trained to discriminate mitragynine (32 mg/kg, i.p., administered 30 min prior to sessions). Immediately following an injection of either the training dose or vehicle, each subject was returned to their home cage for the duration of the pretreatment interval, and were then placed into the operant conditioning chamber. Each training session started with the presentation of both levers and the illumination of the LEDs above each lever. The correct lever was determined by the pre-session injection, i.e., right lever correct after training dose; left lever correct after vehicle. The lever assignments remained the same for that subject for the duration of the study and were counterbalanced among subjects. Each downward deflection the correct lever activated the pellet dispenser; responses on the injection-inappropriate lever had no programmed consequence. Each training session lasted for up to 15 min or until a maximum of 50 pellets was delivered, whichever occurred first. The FR value was increased systematically to 10, i.e., 10 responses on the correct lever were required for pellet delivery. The order of drug and vehicle training followed a double-alternation sequence (i.e. right–left–left–right), with periods of single alternation (i.e. right-left-right-left) irregularly interposed to ensure that drug and vehicle were exerting control over choice behavior.

Drug Discrimination Testing. Test sessions commenced when the following criteria were met individually per rat for four consecutive sessions under the FR10 schedule of reinforcement: 1) A minimum of 80% of the total responses was correct; and 2) the total of incorrect responses made prior to delivery of the first reinforcer was less than 10. All rats in both groups satisfied the test criteria. After the first test session, these criteria needed to be satisfied for one vehicle and one drug training session prior to the next test. The order of training (i.e., drug and vehicle) varied non-systematically between test sessions. Test sessions were identical to training sessions, except that 10 responses in either lever resulted in delivery of food and various doses of drugs were administered. Dose-effect assessments were conducted first for each training drug in all subjects, followed in a non-systematic order by substitution of various compounds for each training drug and pretreatment tests. Doses of test compounds were administered from doses that produced less than group averages of 20% drug-appropriate responding up to doses that produced greater than or equal to group

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averages of 80% drug-appropriate responding, decreased response rate to less than 20% of the vehicle control per subject, or were deemed potentially toxic or could not be increased further due to limitations in solubility. The following drugs were administered in doses increasing by 0.25 log unit 15 min prior to test sessions: morphine (0.32—56 mg/kg), 7-hydroxymitragynine (0.1—17.8 mg/kg), fentanyl (0.0032—0.32 mg/kg), buprenorphine (0.0178—0.56 mg/kg), nalbuphine (1.0—178 mg/kg), U69,593 (0.32—5.6 mg/kg), and SNC 80 (32—100 mg/kg). Mitragynine (3.2—56 mg/kg) and naltrexone (0.032 mg/kg) were administered 30 min prior to sessions. Naltrexone (0.032 mg/kg) was administered alone and in combination with morphine (3.2—56 mg/kg), mitragynine (17.8—56 mg/kg), and 7-hydroxymitragynine (0.32—17.8 mg/kg). The largest dose of mitragynine was 56 mg/kg; 100 mg/kg was lethal even in the presence of 10 mg/kg naltrexone. At the end of the study, the dose-effect functions of each training drug were individually redetermined in all subjects.

Data analyses. To calculate binding affinity, the IC_{50} values were determined using average values from at least three experiments conducted in triplicate and calculated using a nonlinear, least-squares regression analysis (Prism 8; GraphPad Software, Inc., San Diego, CA). IC_{50} values were converted to K_i values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973). Table 1 shows a summary of the present scintillation counting conditions described above. Percent DAMGO-stimulated [^{35}S]GTPγS binding was defined as [(net-stimulated binding by a test compound)/(net-stimulated binding by 10 μM DAMGO)]×100%. For behavioral testing, a within-subjects design and total sample size of 8 (four rats per sex) were used for every experiment. All data are shown as mean values (±SEM) as a function of dose. Statistical analyses were conducted using GraphPad Prism version 8 for Windows (San Diego, CA) and SigmaPlot version 14.0 (Systat Software Inc., San Jose, CA). Comparisons were considered significantly different when $P<0.05$. One- and two-way repeated-measures ANOVAs followed by *post-hoc* Bonferroni *t*-tests were used to analyze the effects of dose, sex, training drug, inter-test session, or assessment order (first versus second dose-effect determination for each training drug). Potencies for morphine and mitragynine are calculated for each sex. For all other drugs, when there was no significant main effect of sex, males and females were combined to calculate

potencies and potency ratios. Significant dose x sex interactions are reported and further assessed with *post-hoc* tests; significant differences are noted by asterisks on the abscissae of figures.

The hotplate latencies were normalized to the percentage of the maximum possible antinociceptive effect (%MPE), using the following formula: %MPE = 100 x (post-injection latency - pre-injection baseline latency) / (maximum latency 60 seconds - pre-injection baseline latency). The percentage of drug-appropriate responding was calculated by dividing the total number of responses on the drug-appropriate lever by the total number of responses on both the drug- and vehicle-appropriate levers. The rate of responding was calculated per animal by dividing the total number of responses by the session time in sec. Values were considered a potentially unreliable indication of lever selection and were not plotted or analyzed when the rate of responding was less than 20% of the control rate of responding for any given subject. When greater than half of the sample size was unreliable as defined in this way, the group average percentage of drug-appropriate responding was not plotted or analyzed. However, all data on response rate and MPEs were plotted and analyzed.

Standard linear regression on the linear portion of the dose-effect function (Snedecor and Cochran, 1967) was used to calculate the ED_{50} value and 95% confidence intervals (CIs) when the mean effect (percentages of drug-appropriate responding, MPE, and reductions in response rate) crossed 50% (e.g. more than 50% of drug-appropriate responding and MPE). To compare potency, potency ratios and corresponding 95% CIs were calculated (Tallarida, 2002). If the 95% CIs of the ED_{50} values did not overlap, or the 95% CIs of the potency ratio of the drug alone or in combination with a pretreated compound (i.e. 0.032 mg/kg naltrexone) did not include 1, then the drugs were considered to have significantly different potencies.

RESULTS

Receptor Binding. The binding affinities of mitragynine and 7-hydroxymitragynine at the opioid receptor subtypes were compared to those of reference opioid receptor ligands (Figure 1). The results obtained here were also compared to published values (Table 2). The δ-, and κ-opioid receptor agonists SNC 80 and U69,593, respectively, were not tested beyond 10 μM due to solubility. DADLE and SNC 80 were more potent to displace bound [^{3}H]DADLE than [^{3}H]U69,593 and [^{3}H]DAMGO, while U69,593 and DAMGO

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were selective for the κ- and μ-opioid receptors, respectively. DADLE had high affinity at MOR [K_i value = 3.29 (95% CIs: 1.96—6.77) nM], whereas SNC 80 had relatively low affinity at MOR [K_i value = 2,760 (1,190—6,930) nM] (Table 2).

Mitragynine displaced bound [^{3}H]DAMGO, [^{3}H]U69,593 and [^{3}H]DADLE in a concentration-dependent manner (Figure 1). The binding affinity of mitragynine at MOR [K_i value = 709 (451—1,130) nM] was at least 89-fold than those of fentanyl [K_i value = 7.96 (6.19-10.3) nM], morphine [K_i value = 4.19 (2.03-11.1) nM] and naltrexone [K_i value =1.84 (1.14-3.03) nM] (Table 2). Mitragynine had the lowest affinity at δ-, and κ-opioid receptors among all compounds tested. Mitragynine had 2.4- and 9.6-fold higher binding affinity at μ- than κ- [K_i value = 1,700 (1,090-2,710) nM] and δ-opioid [K_i value = 6,800 (2,980-15,900) nM] receptors, respectively (Table 2).

7-Hydroxymitragynine displaced bound [^{3}H]DAMGO, [^{3}H]U69,593 and [^{3}H]DADLE in a concentration-dependent manner (Figure 1). The binding affinity of 7-hydroxymitragynine at MOR [K_i value = 77.9 (45.8—152) nM] was at least 7.1-fold lower than the affinities of other reference MOR ligands (Table 2). Among the three opioid receptor subtypes, 7-hydroxymitragynine had 2.8- and 3.1-fold higher affinity at μ- than κ- [K_i value = 220 (162—302) nM] and δ-opioid receptor [K_i value = 243 (168—355) nM], respectively (Table 2). 7-Hydroxymitragynine had 9.1-, 7.7- and 28-fold higher binding affinity than mitragynine at μ-, κ- and δ-opioid receptors, respectively.

[^{35}S]GTPγS Binding. The [^{35}S]GTPγS functional assay at hMOR was used to compare the efficacy and potency of mitragynine and 7-hydroxymitragynine to those of DAMGO, fentanyl, morphine, buprenorphine, nalbuphine, naltrexone, U69,593, and SNC 80 (Figure 2A). DAMGO, fentanyl and morphine were full agonists with % maximum stimulation of 103%, 110% and 92.6%, respectively, with fentanyl being the most potent followed by DAMGO then morphine (Figure 2A). Buprenorphine and nalbuphine were partial agonists, i.e., % maximum stimulation of 42.8% and 22.8%, respectively (Figure 2A). Naltrexone produced no agonism up to 10 μM (Figure 2A). The % maximum stimulation values of DAMGO, fentanyl, morphine, buprenorphine, nalbuphine and naltrexone were similar to reported literature values (Emmerson et al., 1996; Selley et al., 1997). The δ- (SNC 80) and κ-opioid receptor agonists (U69,593) produced 21% and 30%

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stimulation of MOR at 10 μM, respectively. Higher concentrations were not tested due to solubility limitations. Mitragynine did not produce significant agonism up to 100 μM (Figure 2A). 7-Hydroxymitragynine was a partial agonist, i.e., % maximum stimulation of 41.3% (Figure 2A, Table 3). Because the lack of MOR activity of mitragynine was not expected, [^{35}S]GTPγS binding was independently tested at Eurofins Cerep (Celle l'Evescault, France). Mitragynine did not produce agonism up to 30 μM at κ- and μ- and up to 200 μM at δ-opioid receptors (Supplemental Figure 1).

The effects of naltrexone, nalbuphine, buprenorphine, mitragynine and 7-hydroxymitragynine on DAMGO-stimulated [^{35}S]GTPγS binding, and the effects of naltrexone and mitragynine on 7-hydroxymitragynine-stimulated [^{35}S]GTPγS binding, were assessed to explore possible antagonism (Figure panels 2B and 2C). Naltrexone at a 10-fold greater concentration than its K_i value at MOR (18.4 nM, Table 2) produced an 81-fold rightward shift in the DAMGO concentration-effect curve (Figure 2B, Table 4). Mitragynine at 10x its MOR K_i value (7.09 μM, Table 2) produced 33-fold rightward shift in the concentration-effect curve of DAMGO (Figure 2B). Buprenorphine, 7-hydroxymitragynine and nalbuphine at 10x their MOR K_i values antagonized DAMGO 10-, 7-, and 8-fold, respectively (Figure 2B, Table 4). Mitragynine (7.09 μM) and naltrexone (18.4 nM) produced 22- and 69-fold rightward shifts in the 7-hydroxymitragynine concentration-effect curve, respectively (Figure 2C, Table 4). The antagonist effects of mitragynine at the human δ-, κ- and μ-opioid receptors were further tested at Eurofins Cerep (Celle l'Evescault, France) using the [^{35}S]GTPγS functional assay. The IC_{50} values of mitragynine in the presence of a fixed concentration of DPDPE, U69,593, and DAMGO, at δ-, κ- and μ-opioid receptors were 75.7, 4.73 and 10.8 μM, respectively (Supplemental Figures 2—4).

Control Performance. Baseline hotplate response latency determined in animals discriminating either morphine [mean 8.0 (range 6.8 — 8.8) sec] or mitragynine [9.6 (7.4—17.3) sec] did not significantly differ, nor was there a difference by sex (P values ≥ 0.130). The mean (range) number of sessions required to satisfy the testing criteria were 44 (40—73) in morphine-trained rats and 44 (34—56) in mitragynine-trained rats. There was no significant effect of the training drug, sex or training drug x sex interaction (P values ≥ 0.306). Mean (SEM) response rates (responses/second) were 1.0 (0.06) in morphine-trained rats and 0.90 (0.09) in

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mitragynine-trained rats; there was no significant effect of training drug, sex or their interaction (P values ≥ 0.133 and 0.362, respectively).

Effects of Training Compounds. The ED_{50} values, potency ratios and corresponding 95% CIs for discriminative-stimulus, rate-decreasing and antinociceptive effects of all drugs are summarized in Tables 5 and 6 and Supplemental Table 3. In female and male rats discriminating morphine (3.2 mg/kg), vehicle produced a mean (SEM) of 0.25% (0.12%) and 0.15% (0.15%) morphine-appropriate responding, respectively; mean (SEM) response rates normalized to vehicle control were 120% (8.1%) and 110% (1.1%); and hotplate response latencies expressed as MPE were -4.0% (2.4%) and 0.76% (5.0%), respectively (Supplemental Figures 5 and 6, *left*, filled circle and open square above Vehicle). Morphine dose-dependently increased drug-lever responding, decreased response rate, and increased %MPE (Supplemental Figures 5 and 6). The ED_{50} values (95% CIs) for the discriminative-stimulus effects of morphine were 1.6 (0.88 — 2.1) in females and 2.1 (1.8 — 2.6) mg/kg in males (Tables 5 and 6 and Supplemental Table 3). Corresponding values in females and males to decrease response rates were 9.8 (5.0 — 23) and 5.7 (2.6 — 9.4), respectively; for antinociceptive effects, the values were 38 (36 — 41) and 35 (29 — 42) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3).

In female and male rats discriminating mitragynine (32 mg/kg), mean (SEM) drug-lever responding after vehicle was 2.0% (1.3%) and 2.0% (0.93%), respectively; mean (SEM) response rates normalized to vehicle control were 98% (9.4%) and 91% (4.8%), respectively; and mean (SEM) hotplate response latencies expressed as MPE were 4.1% (5.8%) and 5.1% (3.8%), respectively (Supplemental Figures 5 and 6, *right*, filled circle and open square above Vehicle). Mitragynine dose-dependently increased drug-lever responding and decreased response rate; however, no dose of mitragynine was significantly different from vehicle in the hotplate assay (Supplemental Figures 5 and 6). The ED_{50} values (95% CIs) for the discriminative-stimulus effects of mitragynine were 14 (9.0 — 18) mg/kg in females and 17 (14 — 20) mg/kg in males (Tables 5 and 6 and Supplemental Table 3). Corresponding values to decrease response rates were 36 (30 — 46) and 65 (43 — 575) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). The potencies of morphine and

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mitragynine to produce discriminative-stimulus, rate-decreasing and antinociceptive effects did not differ significantly between the first and second determinations (Tables 5 and 6 and Supplemental Table 3).

When sex was analyzed as a main effect, there were no significant differences for discriminative-stimulus, rate-decreasing and antinociceptive effects for morphine (F values $\leq$ 3.37; P values $\geq$ 0.116) and mitragynine (F values $\leq$ 2.03; P values $\geq$ 0.205, Supplemental Table 2).

Cross-Substitution. In rats discriminating morphine, 56 mg/kg of mitragynine produced a maximum of 72% (SEM: 24%) drug-appropriate responding and decreased operant response rates to 30% of vehicle control; %MPE was not increased above 7% by any dose of mitragynine (Figures 3 and 4, *left*, filled circles). The mitragynine ED_{50} values (95% CIs) to increase morphine-lever responding and to decrease response rates were 30 (19 — 60) mg/kg and 46 (36 — 64) mg/kg (Tables 5 and 6 and Supplemental Table 3). In rats discriminating mitragynine, there was no significant effect of sex and no morphine dose x sex interaction for the discriminative-stimulus, rate-decreasing, and antinociceptive effects (Supplemental Table 2). Morphine produced a maximum of 65% (SEM: 20%) mitragynine-lever responding at 17.8 mg/kg; the 56 mg/kg dose of morphine eliminated responding and increased MPE to 90% (SEM: 4.3%) (Figures 3 and 4, *right*, gray squares). The morphine ED_{50} values to increase mitragynine-lever responding, to decrease response rates, and to increase MPE were 16 (10 — 36), 25 (19 — 31), and 36 (31 — 41) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). There was no significant effect of sex and its interaction with morphine dose on mitragynine-lever responding and MPE (Supplemental Table 2). For effects on response rate, there was no significant effect of sex (P=0.620, Supplemental Table 2); however, there was a significant morphine dose x sex interaction (P=0.002, Supplemental Table 2). *Post-hoc* testing indicated significant differences that are shown in Supplemental Figure 6.

Table 7 shows potency ratios comparing morphine and mitragynine between the two training drugs. For all three variables (discriminative-stimulus, rate-decreasing and antinociceptive effects), morphine was at least 8.5-fold more potent in morphine-trained rats than in mitragynine-trained rats. In contrast, the potencies of mitragynine to produce discriminative-stimulus and rate-decreasing effects did not differ between training drugs (Supplemental Table 4).

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Effects of 7-Hydroxymitragynine. In morphine-trained rats, 7-hydroxymitragynine at 1.0 mg/kg produced a maximum of 100% (0.1%) drug-lever responding; 17.8 mg/kg eliminated responding and produced 78% (8.7%) MPE (Figures 5 and 6, *left*, open circles). The ED_{50} values of 7-hydroxymitragynine to increase morphine-lever responding, decrease response rates, and increase MPE were 0.28 (0.077 — 0.41), 4.7 (3.3 — 6.4), and 11 (9.3 — 12) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). In mitragynine-trained rats, 7-hydroxymitragynine produced a maximum of 98% (1.2%) drug-lever responding at 1.78 mg/kg; 17.8 mg/kg decreased response rates to 0.041% of vehicle control and produced 58% (9.7%) MPE (Figures 5 and 6, *right*, open circles). The ED_{50} values of 7-hydroxymitragynine to increase mitragynine-lever responding, decrease response rates, and increase %MPE were 0.42 (0.083 — 0.66), 5.5 (3.9 — 7.4), and 16 (14 — 21) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3).

Effects of Fentanyl. In morphine-trained rats, fentanyl dose-dependently increased drug-lever responding to 98% (0.15%) at 0.056 mg/kg; 0.32 mg/kg decreased response rates to 0.018% of vehicle control and increased MPE to 96% (3.0%) (Figures 5 and 6, *left*, open upward triangles). The ED_{50} values of fentanyl to increase morphine-lever responding, decrease response rates, and increase MPE were 0.022 (0.011 — 0.032), 0.17 (0.15 — 0.20), and 0.14 (0.11 — 0.17) mg/kg, respectively (Tables 5 and 6 and Supplemental table 3). In mitragynine-trained rats, fentanyl produced a maximum of 81% (SEM: 20%) drug-lever responding at 0.178 mg/kg; 0.32 mg/kg decreased responding to 3.0% of vehicle control and increased MPE to 100% (Figures 5 and 6, *right*, open upward triangles). The ED_{50} values of fentanyl to increase mitragynine-lever responding, decrease response rates, and increase MPE were 0.051 (0.012 — 0.091), 0.17 (0.14 — 0.21), and 0.12 (0.093 — 0.14) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). There was a significant fentanyl dose x sex interaction on mitragynine-appropriate responding (Supplemental Table 2). *Post-hoc* testing suggested that fentanyl was more potent in females than males (Supplemental Table 2 and Supplemental Figure 6). None of the effects of fentanyl significantly differed as a function of training drug (Supplemental Table 4).

Effects of U69,593 and SNC 80. In morphine-trained rats, U69,593 produced a maximum of 72% (24%) drug-lever responding at 3.2 mg/kg; 5.6 mg/kg suppressed responding and increased MPE to 52% (13%)

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(Figures 5 and 6, *left*, cross-hatch circles). The U69,593 ED_{50} values to increase morphine-lever responding, decrease response rates, and increase MPE were 2.3 (1.6 — 4.4), 2.3 (1.9 — 2.8), and 5.1 (4.1 — 6.8) mg/kg (Tables 5 and 6 and Supplemental Table 3). There was a significant U69,593 dose x sex interaction for U69,593-induced antinociception. *Post-hoc* testing indicated MPE at 5.6 mg/kg was greater in females than males (Supplemental Table 2). In mitragynine-trained rats, U69,593 produced a maximum of 22% (SEM: 14%) drug-lever responding at 1.78 mg/kg [40% (SEM: 34%) drug-lever responding at 3.2 mg/kg (N=1 per sex)]; 5.6 mg/kg dose of U69,593 markedly decreased response rates and produced a 51% (7.3%) MPE (Figures 7 and 8, *right*, circles with cross hatch). The ED_{50} value of U69,593 to produce the rate-decreasing and antinociceptive effects were 2.5 (2.1 — 3.0) and 5.7 (5.1 — 6.8) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). In morphine-trained rats, SNC 80 produced a maximum of 34% (11%) drug-lever responding at 56 mg/kg; 100 mg/kg decreased response rates to 51% (13%) of vehicle control and increased MPE to 8.6% (5.5%) (Figures 5 and 6, *left*, diamonds with cross hatch). Doses higher than 100 mg/kg were insoluble in the chosen vehicle. In mitragynine-trained rats, SNC 80 produced a maximum of 23% (SEM: 11%) drug-lever responding; 100 mg/kg decreased response rates to 58% (12%) of control and did not significantly increase MPE (Figures 5 and 6, *right*, diamonds with cross hatch). The effects of U69,593 and SNC 80 did not significantly differ as a function of training drug (Supplemental Table 4).

Effects of Buprenorphine and Nalbuphine. In morphine-trained rats, buprenorphine produced a maximum of 100% (0.09%) drug-lever responding at 0.178 mg/kg; 0.56 mg/kg decreased response rates to 31% (14%) of vehicle control and did not significantly increase MPE (Figures 5 and 6, *right*, open downward triangles). The ED_{50} values of buprenorphine to increase morphine-lever responding and decrease response rates were 0.084 (0.046 — 0.12) and 0.34 (0.25 — 0.53) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). In mitragynine-trained rats, the 0.56 mg/kg dose of buprenorphine produced a maximum of 79% (SEM: 20%) drug-lever responding, decreased response rates to 29% (11%) of control, and did not significantly change MPE relative to vehicle in the hot plate assay (Figures 5 and 6, *right*, open downward triangles). The buprenorphine ED_{50} values for increasing mitragynine-lever responding and decreasing response rates were 0.19 (0.067 — 0.31) and 0.40 (0.32 — 0.53) mg/kg, respectively (Tables 5 and 6 and Supplemental 3).

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Buprenorphine did not show any antinociceptive activity here; however, the antinociceptive effects of 0.32 mg/kg buprenorphine at a lower temperature (50°C) was robust (100%) in naïve female and male rats in a preliminary study (data not shown).

In morphine-trained rats, nalbuphine produced a maximum of 99% (0.3%) drug-lever responding at 32 mg/kg; 178 mg/kg decreased response rates to 4.4% of control and produced a 2.9% (5.5%) MPE (Figures 5 and 6, *left*, open diamonds). The ED_{50} values of nalbuphine to increase morphine-lever responding and decrease response rates were 7.3 (2.3 — 11) and 81 (69 — 97) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). In mitragynine-trained rats, nalbuphine produced a maximum of 98% (SEM: 0.9%) drug-lever responding at 56 mg/kg; 178 mg/kg markedly decreased rates and produced a 2.2% (5.1%) MPE (Figures 5 and 6, *right*, open diamonds). The ED_{50} values of nalbuphine to increase mitragynine-lever responding and to decrease response rates were 6.7 (4.3 — 9.0) and 110 (92 — 137) mg/kg, respectively (Tables 5 and 6 and Supplemental Table 3). Nalbuphine did not significantly increase MPE (Figures 5 and 6, *right*, diamonds with cross hatch). The effects of buprenorphine and nalbuphine did not significantly differ as a function of training drug (Supplemental Table 4).

Effects of Naltrexone Combined with Morphine, Mitragynine and 7-Hydroxymitragynine. In morphine-trained rats, naltrexone (0.032 mg/kg) resulted in 0.1% (0.1%) drug-lever responding, 94% (5.9%) of vehicle-control response rates, and 2.5% (4.3%) MPE (Figures 7 and 8, *left*, open squares above Vehicle). When combined with morphine, naltrexone (0.032 mg/kg) produced significant rightward shifts in the dose-response function for the discriminative-stimulus effects of morphine when the control was determined first (8.2-fold) and then re-determined (9.5-fold) (Table 7, Figure 7, *top left*, open squares). In the presence of naltrexone, there was a significant main effect of sex (P=0.049) but not of its interaction with morphine dose (P=0.372) (Supplemental Table 2). *Post-hoc* tests indicated that drug-lever responding at 10 mg/kg of morphine was greater in females than males (Supplemental Table 2). Naltrexone (0.032 mg/kg) produced a smaller (2.0-fold) rightward shift in the morphine dose-effect function for rate-decreasing effects (Table 7, Figure 7, *bottom left*, open squares). In the presence of naltrexone, there was a significant effect of sex (P=0.037) and no significant interaction of morphine dose (P=0.657) x sex for rate-decreasing effects

(Supplemental Table 2). *Post-hoc* testing indicated response rates at 32 mg/kg of morphine were greater in females than males (Supplemental Table 2). For antinociceptive effects, there was no significant main effect of sex for naltrexone in combination with morphine (P=0.370); there was a significant morphine dose x sex interaction (P=0.008), with *post-hoc* testing indicating significantly greater MPE at 56 mg/kg of morphine in females than males (Supplemental Table 2).

In mitragynine-trained rats, naltrexone (0.032 mg/kg, i.p.) resulted in 0.32% (0.21%) drug-lever responding, 92% (12%) of vehicle control response rates, and -6.7% (3.3%) MPE (Figures 7 and 8, *right*, open squares above Vehicle). In the presence of naltrexone, 56 mg/kg of mitragynine produced a maximum of 27% (SEM: 17%) drug-lever responding, decreased response rates to 53% (SEM: 11%) of vehicle controls, and produced -3.9% (4.6%) MPE (Figures 7 and 8, *right*, open squares). The magnitude of the significant antagonism produced by naltrexone on the discriminative-stimulus of mitragynine could not be calculated due to lack of antagonism of the rate-decreasing effects of mitragynine.

In morphine-trained rats, 0.032 mg/kg naltrexone significantly antagonized the ability of 7-hydroxymitragynine to substitute for morphine, shifting its dose-effect function 2.4-fold rightward (Table 7, Figure 7, *top middle*, open squares). Naltrexone, at 0.032 mg/kg, did not significantly antagonize the rate-decreasing effects of 7-hydroxymitragynine (Table 7, Figure 7, *bottom middle*, open squares). Naltrexone (0.032 mg/kg) significantly antagonized the antinociceptive effects of 7-hydroxymitragynine, reducing the MPE of 7-hydroxymitragynine (17.8 mg/kg) alone from 78% (8.7%) to 3.4% (1.9%) (Figure 8, *middle*, open squares).

Effects of Nalbuphine and Mitragynine Combined with Morphine. In morphine-trained rats, nalbuphine (1.78 mg/kg) produced 0.32% (0.23%) morphine-appropriate responding, 109% (8.5%) of vehicle-control response rates, and a -2.9% (1.7%) MPE (Figures 9 and 10, *left*, open squares above Vehicle). The same dose of nalbuphine significantly increased the potency of morphine to produce discriminative-stimulus effects, evidenced by a 4.7-fold leftward in the morphine discrimination dose-effect function (Table 7, Figure 9, *top left*, open squares). In contrast, in the same animals and during the same experimental sessions, nalbuphine significantly decreased the potency of morphine to decrease response rates and increase MPE; the morphine

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dose-effect functions were shifted rightward 1.9- and greater than 1.6-fold, respectively (Table 7, Figure 9, *bottom left* and Figure 10, *left*, open squares). Mitragynine (5.6 mg/kg) produced 0.64% (0.37%) morphine-lever responding, 93% (6.9%) of vehicle-control response rates, and 7.0% (6.5%) MPE (Figures 9 and 10, *right*, open squares above Vehicle). The same dose of mitragynine significantly increased the potency of morphine 3.3-fold (Table 6, Figure 9, *top right*, open squares). In contrast, and in the same animals during the same experimental sessions, mitragynine (5.6 mg/kg) antagonized the antinociceptive effects of morphine (Figure 10, *right*, open squares) and did not significantly modify the morphine dose-effect function for rate-decreasing effects (Table 7, Figure 9, *bottom right*, open squares).

DISCUSSION

Pharmacological mechanisms of two kratom alkaloids, mitragynine and 7-hydroxymitragynine, were assessed 1) in cell membranes expressing hMOR and 2) behavioral assays sensitive to MOR agonism in rats, i.e., morphine discrimination and antinociception. Mitragynine had lower MOR binding affinity than 7-hydroxymitragynine; mitragynine lacked intrinsic activity (efficacy), i.e., was a MOR antagonist, whereas 7-hydroxymitragynine was a MOR partial agonist. In rats, the same apparent rank order of efficacy was evident (i.e., mitragynine < 7-hydroxymitragynine); behavioral effects were apparently more sensitive to MOR agonism than the [^{35}S]GTPγS assay. Mitragynine was established as a discriminative-stimulus, naltrexone antagonized the discriminative-stimulus effects of both mitragynine and 7-hydroxymitragynine, and mitragynine functioned as both an agonist and antagonist depending on the efficacy required, i.e., when the efficacy requirement was low (drug discrimination) mitragynine was an agonist, and when the efficacy requirement was high (antinociception) mitragynine was an antagonist.

Mitragynine is typically the most abundant alkaloid in kratom products, and 7-hydroxymitragynine is a common mitragynine metabolite. The extent to which potential differences in their MOR efficacy translate to behavioral effects is currently unknown. MOR efficacy is a critical determinant of the therapeutic and adverse effects of MOR agonists, and low efficacy MOR agonists are clinically safer than higher efficacy MOR agonists. Using [^{35}S]GTPγS stimulation at hMOR to assess efficacy, we found that mitragynine was a MOR antagonist, and 7-hydroxymitragynine was a MOR partial agonist. Both mitragynine and 7-

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hydroxymitragynine were MOR partial agonists in previous studies (Kruegel et al., 2016; Varadi et al., 2016; Obeng et al., 2020). Differences in the efficacy of mitragynine across studies could reflect differences in receptor reserve and MOR-G protein coupling efficiency (Niedernberg et al., 2003). Previous studies used different species: hMOR (Kruegel et al., 2016; Obeng et al., 2020) versus rodent MOR (Varadi et al., 2016). The experimental techniques have differed: bioluminescence resonance energy transfer and homogeneous time resolved fluorescence (Kruegel et al., 2016; Varadi et al., 2016) versus [^{35}S]GTPγS stimulation.

In contrast to mitragynine exhibiting no efficacy at hMOR in the current study, its behavioral effects were largely consistent with MOR agonism. Mitragynine and morphine exhibited numerous similarities in our drug discrimination assays. In both assays, MOR agonists (buprenorphine, nalbuphine, 7-hydroxymitragynine and fentanyl) produced high levels of drug-lever responding. The rank order of hMOR binding affinities of fentanyl, 7-hydroxymitragynine, morphine, nalbuphine and mitragynine were similar to their ED_{50} values in the discrimination assays. Buprenorphine was an exception, i.e., exhibited highest affinity and the second lowest ED_{50} value. The KOR agonist U69,593 and the DOR agonist SNC 80 produced less drug-lever responding than the MOR agonists, further underscoring predominant actions of the training drugs at MOR. The incomplete substitution of U69,593 and SNC 80 for morphine may reflect very low MOR efficacy at relatively high doses and the high sensitivity of the relatively low training dose of morphine to MOR agonism (see Picker et al., 1990). SNC 80 doses greater than 100 mg/kg could not be dissolved in our chosen vehicle; however this appears to be a behaviorally active dose as evidenced by antinociceptive effects in drug-naive rats (Craft et al., 2001). The vast majority of morphine discrimination assays are selectively mediated by MOR agonism (Picker et al., 1990; Walker and Young, 2001), and it appears MOR agonism is the predominant mechanism by which mitragynine produces discriminative-stimulus effects. The qualitatively distinct effects of mitragynine *in vitro* (no agonism in our study) and *in vivo* (agonism) might be due to metabolism of mitragynine to 7-hydroxymitragynine *in vivo* (Kruegel et al., 2019; Hiranita et al., 2020; Kamble et al., 2020).

Mitragynine did not completely substitute for the morphine discriminative-stimulus and vice versa. The discriminative-stimulus effects of mitragynine may not be solely mediated by MOR and substitution may

have been limited by rate-decreasing effects stemming from this additional pharmacological activity. When two receptor types differentially mediate the discriminative-stimulus effects of a training drug (i.e., due to higher binding affinity and/or efficacy at one site versus another), actions at the lower affinity site can be detected by systematically increasing the training dose (e.g., nicotine; Jutkiewics et al., 2011). By contrast, training drugs acting selectively at a receptor type will generally exhibit discriminative-stimulus effects that are mediated predominantly if not exclusively by that receptor type across a range of training doses. Morphine discriminations are selectively mediated by MOR regardless of training dose, and MOR efficacy is a key determinant of substitution: as the training dose of morphine is increased, higher MOR efficacy is required for substitution (Young et al., 1992). It is presently unclear whether multiple receptor sites of action might be detected by varying the training dose of mitragynine. Mitragynine was trained as a discriminative-stimulus at a training dose of 15 mg/kg (Harun et al., 2015), which is lower than the current training dose (32 mg/kg). In comparison to the current results, morphine and mitragynine more completely cross-substituted for one another in the previous study (Harun et al.; 2015), perhaps reflecting greater selectivity of the lower training dose at MOR. Mitragynine binds with moderate affinity at several targets (e.g., α-adrenergic receptors; Boyer et al., 2008; Obeng et al., 2020), and activity at these or other non-opioid receptors could limit the degree of cross-substitution between mitragynine and morphine.

Morphine, fentanyl, 7-hydroxymitragynine, and U69,593 produced antinociception, whereas mitragynine, buprenorphine, nalbuphine and SNC 80 did not. Mitragynine was also previously ineffective against acutely applied noxious heat in rats (see also Hiranita et al., 2019). In contrast, mitragynine produced antinociceptive effects in mice (Matsumoto et al., 1996a; 1996b; Shamima et al., 2012; Kruegel et al., 2019). The rats in our study had a history of repeated MOR agonist treatment; we predict this conferred tolerance, a loss of receptor reserve, an increase in the MOR efficacy required for agonism, the greatest degree of tolerance to lower efficacy MOR agonists, and the greatest loss of sensitivity to effects requiring high efficacy, such as antinociception (Allen and Dykstra, 2000; Barrett et al., 2001; Walker and Young, 2001). In contrast, the efficacy required for drug discrimination is low, e.g., low training doses of morphine (3.2 mg/kg) are sensitive to low efficacy agonists (e.g., Young et al., 1991; present results). Here we extended

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these findings to include substitution of mitragynine for morphine. Doses of nalbuphine and mitragynine that did not substitute for morphine increased the potency of morphine to produce discriminative-stimulus effects. However, in the same animal at the same time the same doses of nalbuphine and mitragynine antagonized the antinociceptive effects of morphine. These simultaneous and opposing pharmacological effects of low efficacy MOR agonists are striking: they are agonists when low efficacy is required and antagonists when high efficacy is required. This highlights their therapeutic value: they are not only effective opioid substitution therapies, but they also antagonize the effects of higher efficacy agonists such as respiratory depression and abuse liability. Our results strongly suggest that mitragynine exhibits this profile.

Both nalbuphine and mitragynine produced a rightward shift in the concentration-effect curve of DAMGO in the [^{35}S]GTPγS assay. Partial agonists can produce a rightward shift in the concentration-effect curve of high efficacy MOR agonists; here the partial agonist 7-hydroxymitragynine antagonized DAMGO-induced stimulation of [^{35}S]GTPγS. The small difference in the substitution of mitragynine and nalbuphine in morphine discriminative-stimulus may reflect greater involvement of non-opioid receptors in the actions of mitragynine (Boyer et al., 2008; Ellis et al., 2020; Obeng et al., 2020) as compared to nalbuphine.

A MOR-preferential dose (0.032 mg/kg) of naltrexone (Millan, 1989) antagonized the discriminative-stimulus effects of mitragynine and morphine as well as substitution of 7-hydroxymitragynine for morphine. In a previous study, naloxone reportedly did not antagonize the discriminative-stimulus effects of mitragynine (Harun et al., 2015). In mice, however, naloxone blocked the antinociceptive effects of mitragynine (Matsumoto et al., 1996b). Both naltrexone and naloxone typically antagonize the effects of MOR agonists (Tanda et al., 2016). In the present study, the magnitude of the naltrexone-induced shift in the mitragynine dose-effect function could not be calculated because 100 mg/kg of mitragynine disrupted behavior and was lethal in a subset of animals even in the presence of naltrexone. The failure of naltrexone to antagonize the rate-decreasing effects of mitragynine has been reported previously (Hiranita et al., 2019). The differential antagonism of discriminative-stimulus and rate-decreasing effects evident for not only mitragynine, but also 7-hydroxymitragynine and morphine, suggests that MOR agonists engage naltrexone-insensitive receptors to disrupt operant responding.

In summary, mitragynine was demonstrated to be a low affinity MOR ligand that exerted both agonist and antagonist activity in a predictable manner depending on the efficacy requirements of each assay. Mitragynine binds with moderate affinity to additional receptor types such as α_2-adrenergic receptors (Boyer et al., 2008; Obeng et al., 2019), and it functioned as an α_2-adrenergic agonist in mice using a hotplate assay (Matsumoto et al., 1996a). The present results strongly suggest that mitragynine is a low efficacy MOR agonist *in vivo*, with additional actions at non-opioid receptors. Low efficacy at MOR combined with additional pharmacological mechanism(s) appears to distinguish mitragynine as a unique molecule with considerable potential as an effective therapeutic.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Displacement of radioligands for opioid receptor subtypes. Ordinates: Percentage of specific radiotracer bound to membrane preparations. Abscissae: Concentrations of each competing compound (log scale). *Left*: displacement of [^{3}H]DAMGO labeling MORs. *Middle*: displacement of [^{3}H]U69,593 labeling KORs. *Right*: displacement of [^{3}H]DADLE labeling DORs. Each data point represents the mean results of three repeated experiments; vertical bars represent SEMs (N≥3) from at least three independent triplicate replications per sample. Ki and 95%CI values from curve-fitting analyses of these data are shown in Table 2.

Figure 2. [^{35}S]GTPγS stimulation in CHO cell lines stably expressing the hMORs. Ordinates: Percentage of maximum stimulation of [^{35}S]GTPγS binding normalized to maximum DAMGO response as 100%. Abscissae: Concentrations of each test compound (log scale). *Panel A*: Effects of test compounds alone. *Panel B*: Effects of DAMGO in combination with buffer, 110 nM (10 × K_i value at hMOR) nalbuphine, 18.4 nM (10 × K_i value at hMOR) naltrexone, 7.09 μM (10 × K_i value at hMOR) mitragynine, 779 nM (10 × K_i value at hMOR) 7-hydroxymitragynine, and 9.03 nM (10 × K_i value at hMOR) buprenorphine. *Panel C*: Effects of 7-hydroxymitragynine in the presence of buffer, 18.4 nM (10 × K_i value at hMOR) naltrexone, and 7.09 μM (10 × K_i value at hMOR) mitragynine. Data are percentages of the mean ± SEM (N≥3 per data point) of net stimulated [^{35}S]GTPγS binding divided by stimulation produced by 10 μM DAMGO. The results were selected from at least three independent triplicate replications per sample for all panels. EC_{50} and E_{max} values from curve-fitting analyses of these data are shown in Table 3.

Figure 3. Mitragynine substitution in rats trained to discriminate morphine (*left*), and morphine substitution in rats trained to discriminate mitragynine (*right*). Abscissae: Vehicle and drug dose in mg/kg (i.p., log scale). Ordinates: *Top*, percentage of responses on the training drug-appropriate lever. *Bottom*, mean rates of responding expressed as a percentage of vehicle control. Morphine and mitragynine were administered i.p., respectively, at 15 and 30 min before sessions. Each point represents the mean ± SEM (N=8) except for % Drug Lever at 56 mg/kg mitragynine in the morphine discrimination (N=4) and 17.8 mg/kg morphine in the

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mitragynine discrimination (N=6) mg/kg. Details for statistical analyses are shown in Tables 5 and 6 and Supplemental Tables 2—4.

Figure 4. Antinociceptive effects determined in conjunction with the discrimination tests shown in Figure 5. Abscissae: Vehicle and drug dose in mg/kg (i.p., log scale) in separate groups of rats discriminating either morphine (*left*) or mitragynine (*right*). Ordinates: percentage of maximum possible antinociceptive effects. Each point represents the mean ± SEM (N=8). Morphine and mitragynine were administered i.p., respectively, at 15 and 30 min before sessions. Details for statistical analyses are shown in Tables 5 and 6 and Supplemental Tables 2—4.

Figure 5. Substitution tests in separate groups of rats discriminating either morphine (*left*) or mitragynine (*right*). Abscissae: Vehicle and drug dose in mg/kg (i.p., log scale). Ordinates: *Top*, percentage of responses on the training drug-appropriate lever. *Bottom*, mean rates of responding expressed as a percentage of vehicle control. All compounds were administered i.p. 15 min before sessions except mitragynine (30 min prior to sessions). The training drug dose-effect functions are replotted from Figure 5. Each point represents the mean ± SEM (N=8) except in the morphine discrimination [7-hydroxymitragynine at 0.56, 1.0 mg/kg and 1.78 mg/kg (N=7) and 3.2 mg/kg (N=5) mg/kg, buprenorphine at 0.178 mg/kg (N=5) mg/kg, nalbuphine at 100 mg/kg (N=7) mg/kg, U69,593 at 1.0 mg/kg (N=7), 1.78 mg/kg (N=6), and 3.2 mg/kg (N=4), and SNC 80 at 100 mg/kg (N=7) mg/kg] and in the mitragynine discrimination [7-hydroxymitragynine at 1.0 and 1.78 mg/kg (N=7), fentanyl at 0.1 mg/kg (N=7) and 0.178 mg/kg (N=5), buprenorphine at 0.32 mg/kg (N=7) and 0.56 mg/kg (N=5), nalbuphine at 100 mg/kg (N=6), and U69,593 at 1.0 and 1.78 mg/kg (N=7)]. Details for statistical analyses are shown in Tables 5 and 6 and Supplemental Tables 2—4.

Figure 6. Antinociceptive effects determined in conjunction with the discrimination tests shown in Figure 7. Abscissae: Vehicle and drug dose in mg/kg (i.p., log scale). Ordinates: percentage of maximum possible antinociceptive effects. Each point represents the mean ± SEM (N=8). All compounds were administered i.p. 15 min before sessions except mitragynine (30 min prior to sessions). The morphine and mitragynine dose-effect functions are replotted from Figure 4 *left* and *right*, respectively. Details for statistical analyses are shown in Tables 4 through 7 and Supplemental Table 3.

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Figure 7. Naltrexone antagonism of the effects of morphine and 7-hydroxymitragynine in rats discriminating morphine, and mitragynine in rats discriminating mitragynine. Abscissae: Vehicle and dose in mg/kg (i.p., log scale) for mitragynine (*left*), 7-hydroxymitragynine (*middle*) and mitragynine (*right*). Ordinates: *Top*, percentage of responses on the training drug-appropriate lever. *Bottom*, mean rates of responding expressed as a percentage of vehicle control. Morphine and 7-hydroxymitragynine were administered i.p. 15 min before sessions and mitragynine and naltrexone (0.032 mg/kg) were administered i.p. 30 min before sessions. The dose-effect functions for the training drugs morphine and mitragynine are replotted from Figure 5, and the dose-effect function for mitragynine is replotted from Figure 7. Each point represents the mean ± SEM (N=8) except for naltrexone + morphine at 17.8 mg/kg (N=7) and 32 mg/kg (N=5), naltrexone + 7-hydroxymitragynine at 1.78 mg/kg (N=6) and 3.2 mg/kg (N=4) mg/kg, naltrexone + mitragynine at 56 mg/kg (N=7). Details for statistical analyses are shown in Table 5 and 6 and Supplemental Tables 2—4.

Figure 8. Antinociceptive effects determined in conjunction with the discrimination tests shown in Figure 9. Abscissae: Vehicle and drug dose in mg/kg (i.p., log scale). Ordinates: percentage of maximum possible antinociceptive effects. Each point represents the mean ± SEM (N=8). Morphine and 7-hydroxymitragynine were administered i.p. 15 min before sessions and mitragynine and naltrexone (0.032 mg/kg) were administered i.p. 30 min before sessions. The morphine and mitragynine dose-effect functions are replotted from Figure 6 *left* and *right*, respectively, and the 7-hydroxymitragynine dose-effect function is replotted from Figure 8. Details for statistical analyses are shown in Tables 5 and 6 and Supplemental Tables 2—4.

Figure 9. Discriminative-stimulus effects of morphine in combination with nalbuphine (*left*) and mitragynine (*right*). Abscissae: Vehicle and morphine dose in mg/kg (i.p., log scale). Ordinates: *Top*, percentage of responses on the morphine-appropriate lever. *Bottom*, mean rates of responding expressed as a percentage of vehicle control. Morphine and nalbuphine (1.78 mg/kg) were administered i.p. 15 min before sessions while mitragynine (5.6 mg/kg) was administered i.p. 30 min before sessions. The morphine dose-effect function is replotted from Figure 5. Each point represents the mean ± SEM (N=8) except for nalbuphine + morphine at 17.8 mg/kg (N=7), and mitragynine + morphine at 10 mg/kg (N=5). Details for statistical analyses are shown in Table 5s and 6 and Supplemental Tables 2—4.

Figure 10. Antinociceptive effects determined in conjunction with the discrimination tests shown in Figure 11. Abscissae: Vehicle and drug dose in mg/kg (i.p., log scale). Ordinates: percentage of maximum possible antinociceptive effects. Each point represents the mean ± SEM (N=8). Morphine and nalbuphine were administered i.p. 15 min before sessions and mitragynine was administered i.p. 30 min before sessions. The morphine dose-effect function is replotted from Figure 6. Details for statistical analyses are shown in Tables 5 and 6 and Supplemental Tables 2—4.

TABLES

Table 1 Summary of scintillation counting conditions employed for assessing affinity at various binding sites in competition for the radioligands labeling human opioid receptor subtypes. K_d and B_{max} values in parentheses are 95% confidence intervals (95% CIs).

Receptor	Source (cell)	Radioligand	Radioligand concentration (nM), (Mean ± SEM)	Nonspecific Binding (10 μM)	Incubation Buffer	Incubation Time (Room Temperature)	K_d (nM) (95% CI)	B_{max} (pmol/mg) (95% CI)
DOR	CHO	[^{3}H]DADLE	0.864 ± 0.035	SNC 80	TME buffer	60 min	0.426 (0.272—0.580)	5.04 (4.54—5.53)
KOR	HEK-293	[^{3}H]U69,593	1.60 ± 0.139	U69,593	TME buffer	60 min	1.44 (0.453—2.42)	4.98 (4.13—5.83)
MOR	CHO	[^{3}H]DAMGO	1.18 ± 0.211	Naltrexone	TME buffer	60 min	1.72 (0.652—2.79)	6.41 (5.07—7.74)

Table 2 Inhibition of binding of the radioligands labeling opioid receptor subtypes. Values are K_i values, except as indicated, for displacement of the listed radioligands. Values in parentheses are 95% CIs. Values listed from previous studies were also added as reference. EC_{50} and E_{max} values from curve-fitting analyses of these data are shown in Figure 1.

Compound	μ K_i (95% CIs) nM	δ K_i (95% CIs) nM	κ K_i (95% CIs) nM	κ/μ	δ/μ	δ/κ
Buprenorphine	0.903 (0.71—1.21)	1.51 (0.975—2.35)	1.29 (0.980—2.09)	1.43	1.67	1.17
DADLE	3.29 (1.96—6.77)	0.426 (0.272—0.580)[a]	3050 (2020—4650)	927	0.129	0.000140
DAMGO	4.15[a] (1.85—13.1)	880 (442—1,930)	1,200 (556—2,770)	289	212	0.733
Fentanyl	7.96 (6.19—10.3)	539 (300—987)	202 (128—349)	25.4	67.7	2.66
7-Hydroxymitragynine	77.9 (45.8—152) 37 (SEM: 4, mouse)[b] 47 (SEM: 18, human)[c] 7.16 (SEM: 0.94, human)[d] 70 (human)[e]	243 (168—355) 91 (SEM: 8, mouse)[b] 219 (SEM: 41, human)[c] 236 (SEM: 6, human)[d]	220 (162—302) 132 (SEM: 7, mouse)[b] 188 (SEM: 38, human)[c] 74.1 (SEM: 7.8, human)[d]	2.82 3.57 (mouse)[b] 4 (human)[c] 10.4 (human)[d] 4.57 (human)[e]	3.12 2.46 (mouse)[b] 4.66 (human)[c] 33.0 (human)[d] 6.71 (human)[e]	1.15 0.69 (mouse)[b] 1.16 (human)[c] 3.2 (human)[d] 1.47 (human)[e]

		470 (human)[e]	320 (human)[e]			
Mitragynine	709 (451—1,130) 230 (SEM: 47, mouse)[b] 233 (SEM: 48, human)[c] 502 (SEM: 19.4, rat)[f] 7.24 (SEM: 3.44, guinea pig)[g] 740 (human)[e]	6,800 (2,980—15,900) 1,010 (SEM: 50, mouse)[b] >10,000 (human)[c] 7,910 (SEM: 1,140, rat)[f] 60.3 (SEM: 23.1, guinea pig)[g] 6,500 (human)[e]	1,700 (1,090—2,710) 231 (SEM: 21, mouse)[b] 772 (SEM: 207, human)[c] 1,200 (SEM: 79.7, rat)[f] 1,100 (SEM: 436, guinea pig)[g] 1,300 (human)[e]	2.40 1.00(mouse)[b] 3.31[c] (mouse) 2.39 (human)[c] 152 (guinea pig)[f] 1.76 (human)[e]	9.60 4.39 (mouse)[b] Not determined (human)[c] 15.8 (rat)[c] 8.33 (guinea pig)[f] 8.78 (human)[e]	4.00 4.37 (mouse)[b] Not determined (human)[c] 6.59 (rat)[c] 0.0548 (guinea pig)[f] 5 (human)[e]
Morphine	4.19 (2.03—11.1)	250 (177—346)	40.4 (23.7—70.9)	9.64	59.6	6.19
Nalbuphine	11.0 (9.11—13.3)	146 (88.3—242)	13.0 (10.6—16.1)	1.18	13.2	11.2
Naltrexone	1.84 (1.14—3.03)	37.2 (26.3—53.0)	1.19 (0.803—1.79)	0.65	20.2	31.3
SNC 80	2,760 (1,190—6,930)	34.6 (26.5—45.5)	2,020 (1,050—3,950)	0.73	0.013	0.018
U69,593	3,180	6,700	1.62[a] (1.02—2.64)	0.0005	2.11	4,140

	(1,050—11,600)	(2,160—28,000)				

[a]Kd values obtained by homologous competition experiments.

[b][^{125}I]BNtxA for all three opioid receptor subtypes was used in CHO cells expressing mouse opioid receptors (Varadi et al., 2016).

[c][^{125}I]BNtxA for all three opioid receptor subtypes was used in CHO cells expressing human opioid receptors (Kruegel et al., 2016).

[d]The same radioligands as the present study were used but the cell lines used were human HEK-293 cells for MOR and rat basophilic leukemia (RBL) cells for other receptor subtypes (Obeng et al., 2020).

[e][^{3}H]DAMGO, [^{3}H]U69,593, and [^{3}H]DADLE were used in HEK-293 cells expressing human μ-, κ-, and δ-opioid receptors, respectively. (Ellis et al., 2020).

[f][^{3}H]DAMGO, [^{3}H]U69,593, and [^{3}H]DADLE were used in rat whole brain tissue excluding the cerebellum (Yue et al., 2018).

[g][^{3}H]DAMGO, [^{3}H]U69,593, and [^{3}H]DPDPE were used in guinea pig whole brain tissue excluding the cerebellum (Takayama et al., 2002).

Table 3 *In vitro* functional results from the [^{35}S]GTPγS functional assay in transfected CHO cells expressing cloned hMORs. EC_{50} and E_{max} values in parentheses are 95% CIs (unless noted) from curve-fitting analyses of these data shown in Figure 2.

Compound	EC_{50} (95% CI) nM	E_{max} [%DAMGO] (95% CI)
Buprenorphine	16.1 (6.53—39.6)	42.8 (37.2—48.4)
DAMGO	34.8 (24.9—48.6)	103 (96.4—109)
Fentanyl	27.8 (22.6—34.2)	110 (106—115)
7-Hydroxymitragynine	43.4 (25.5—73.8) 53 (SEM: 4)[a] 7.65 (SEM: 0.884)[b]	41.3 (37.1—45.6) 77 (SEM: 5)[a] 96.8 (SEM: 1.8)[b]
Mitragynine	Not determined in the present study 203 (SEM: 13)[a] 320 (SEM: 14.7)[b]	3.46 ((-0.047—6.97) 65 (SEM: 2.8)[a] 44.1 (SEM: 0.62)[b]
Morphine	125 (84.8—184)	92.6 (85.8—99.4)
Nalbuphine	5.87 (4.18—8.23) 9.0 (SEM: 1.6)[c], 1.86 (SE: 0.1)[d]	22.8 (21.1—24.6) 16 (SEM: 0.4)[c], 12[d]
Naltrexone	Not determined in the present study	4.69 (0.162—9.22)
SNC 80	Not determined	20.7 (SEM: 3.01) at 10 μM

U69,593	Not determined	30.1 (SEM: 2.88) at 10 μM

[a][^{35}S]GTPγS functional assay in transfected CHO cells stably expressing cloned mouse MORs (Varadi et al., 2016).

[b]Homogeneous Time Resolved Fluorescence (HTRF) cAMP functional assay in transfected CHO cells stably expressing cloned hMORs (Obeng et al., 2020).

[c][^{35}S]GTPγS functional assay in transfected CHO cells stably expressing cloned mouse MORs (Selley et al., 1998).

[d][^{35}S]GTPγS functional assay in transfected C6 Glioma cells stably expressing cloned rat MORs (Emmerson et al., 1996).

Table 4 *In vitro* functional results for pretreatment with antagonists or partial agonists using the [^{35}S]GTPγS functional assay in transfected CHO cells expressing cloned hMORs. EC_{50} and E_{max} values in parentheses are 95% CIs from curve-fitting analyses of these data shown in Figure 2.

Compound	EC_{50} (95% CI) nM	E_{max} [%DAMGO] (95% CI)	Potency ratio (versus DAMGO or 7-hydroxymitragynine)
DAMGO	34.8 (24.9—48.6)	103 (96.4—109)	
DAMGO + Naltrexone	2,820 (1,650—4,820)	107 (90.7—123)	81.0 (34.0—194)
DAMGO + Mitragynine	1,160 (656—2,050)	105 (90.0—119)	33.3 (13.5—82.3)
DAMGO + Nalbuphine	289 (16.6—5,030)	118 (66.3—171)	8.30 (0.34—202)
DAMGO + 7-Hydroxymitragynine	235 (27.1—2,030)	134 (94.2—173)	6.75 (0.56—81.5)
DAMGO + Buprenorphine	360 (52.9—2,450)	131 (94.0—168)	10.3 (1.09—98.4)
7-Hydroxymitragynine	43.4 (25.5—73.8)	41.3 (37.1—45.6)	
7-Hydroxymitragynine + Naltrexone	3,010 (933—97,30)	46.1 (32.4—59.8)	69.4 (12.6—381)
7-Hydroxymitragynine + Mitragynine	960 (287—3,180)	51.9 (39.6—64.1)	22.1 (3.89—125)

Table 5 ED_{50} values (95% CIs) for the discriminative-stimulus, rate-decreasing, and antinociceptive effects of various compounds in rats trained to discriminate 3.2 mg/kg morphine as shown in Figures 3—10 and Supplemental Figures 5—7. The sample sizes are described in each figure legend. Each value is a combination of females and males unless otherwise noted. For each training drug, potency ratios (95% CIs) are calculated by dividing the ED_{50} values for producing rate-decreasing or antinociceptive effects by the ED_{50} values for producing discriminative-stimulus effects. ND: Not determined *due to lethality; **due to an adverse reaction (scratching behavior), ***due to insolubility in the chosen vehicle, #First assessment, ##Reassessment.

Test drug	ED_{50} (95% CIs)			Potency Ratio	
	Discrimination	Response Rate	Maximum Possible Effect	Rate-Decreasing / Discrimination	Antinociceptive / Discrimination
7-hydroxymitragynine	0.275 (0.0768 — 0.411)	4.73 (3.25 — 6.35)	10.6 (9.26 — 12.3)	17.2 (7.91 — 82.7)	38.5 (22.5 — 160)
7-hydroxymitragynine + 0.032 mg/kg naltrexone	0.670 (0.602 — 0.744)	6.09 (4.78 — 7.53)	ND* [≤ -2.81% (2.03%) @ 32 mg/kg]	9.09 (6.42 — 12.5)	Not Applicable
Buprenorphine	0.0844 (0.0456 — 0.122)	0.343 (0.249 — 0.533)	ND* [≤ -2.81% (2.03%) @ 0.56 mg/kg]	4.06 (2.04 — 11.7)	Not Applicable
Fentanyl	0.0220 (0.0111 — 0.0318)	0.173 (0.150 — 0.202)	0.139 (0.112 — 0.166)	7.86 (4.72 — 18.2)	6.32 (3.52 — 15.0)
Mitragynine	29.6 (18.8 — 55.9)	45.7 (36.4 — 64.1)	ND* [≤7.01% (6.50%) @ 5.6 mg/kg]	1.54 (0.651 — 3.41)	Not Applicable

Morphine	#1.85 (1.54 — 2.20), ##1.60 (1.30 — 1.91)	#13.7 (10.6 — 16.8), ##21.3 (16.8 — 26.2)	#35.2 (32.6 — 38.1), ##33.4 (30.9 — 36.4)	#7.41 (4.82 — 10.9), ##13.3 (8.80 — 20.2)	#19.0 (21.2 — 24.7), ##20.9 (16.2 — 28.0)
Morphine + 0.032 mg/kg naltrexone	15.2 (12.7 — 17.9)	35.4 (28.2 — 47.2)	ND** [≤3.35% (1.92%) @ 56 mg/kg]	2.33 (1.58 — 3.72)	Not Applicable
Morphine + 1.78 mg/kg Nalbuphine	0.335 (0.202 — 0.444)	26.0 (22.4 — 30.3)	ND** [≤27.4% (4.95%) @ 56 mg/kg]	77.6 (50.5 — 150)	Not Applicable
Morphine + 5.6 mg/kg Mitragynine	0.478 (0.265 — 0.503)	11.7 (9.33 — 14.2)	ND** [≤40.6% (6.92%) @ 56 mg/kg]	24.5 (18.6 — 53.6)	Not Applicable
Nalbuphine	7.29 (2.33 — 11.0)	81.3 (68.9 — 96.8)	ND*** [≤2.86% (5.49%) @ 178 mg/kg]	11.2 (6.26 — 41.5)	Not Applicable
SNC 80	ND*** [≤34.3% (11.3%) @ 56 mg/kg]	ND*** [≤51.2% (13.2%) @ 100 mg/kg]	ND*** [≤8.63% (5.47%) @ 100 mg/kg]	Not Applicable	Not Applicable
U69,593	2.29 (1.64 — 4.37)	2.30 (1.85 — 2.77)	5.05 (4.12 — 6.76)	1.00 (0.423 — 0.634)	2.21 (0.943 — 4.12)

Table 6 ED_{50} values (95% CIs) for the discriminative-stimulus, rate-decreasing, and antinociceptive effects of various compounds in rats trained to discriminate either 32 mg/kg mitragynine as shown in Figures 3—10 and Supplemental Figures 5—7. The sample sizes are described in each figure legend. Each value is a combination of females and males unless otherwise noted. For each training drug, potency ratios (95% CIs) are calculated by dividing the ED_{50} values for producing rate-decreasing or antinociceptive effects by the ED_{50} values for producing discriminative-stimulus effects. ND: Not determined *due to lethality; ***due to insolubility in the chosen vehicle, ****due to an adverse reaction (skin ulcer), #First assessment, ##Reassessment.

Test drug	ED_{50} (95% CIs)			Potency Ratio	
	Discrimination	Response Rate	Maximum Possible Effect	Rate-Decreasing / Discrimination	Antinociceptive / Discrimination
7-hydroxymitragynine	0.415 (0.0832 — 0.656)	5.54 (3.92 — 7.35)	16.4 (13.9 — 20.7)	13.3 (5.98 — 88.3)	39.5 (21.2 — 249)
Buprenorphine	0.186 (0.0672 — 0.311)	0.400 (0.322 — 0.534)	ND**** [≤1.40% (6.09%) @ 0.056 mg/kg]	2.15 (1.04 — 7.95)	Not Applicable
Fentanyl	0.0507 (0.0124 — 0.0906)	0.171 (0.140 — 0.214)	0.118 (0.0928 — 0.143)	3.37 (1.55 — 17.3)	2.33 (1.02 — 11.5)
Mitragynine	#15.1 (12.7 — 17.6), ##12.8 (9.31 — 16.0)	#46.2 (38.1 — 60.6), ##47.2 (38.2 — 65.5)	ND* [up to #10.0% (3.32%) and ##8.01% (4.80%) @ 5.6 mg/kg]	#3.06 (2.16 — 4.77), ##3.69 (2.39 — 7.04)	Not Applicable
Mitragynine + 0.032 mg/kg naltrexone	ND* [≤27.2% (17.1%) @ 56 mg/kg]	ND* [≤53.2% (10.8%) @ 56 mg/kg]	ND* [≤ -3.91% (4.56%) @ 56 mg/kg]	Not Applicable	Not Applicable
Morphine	15.7 (10.4 — 36.3)	24.8 (19.3 — 30.5)	35.6 (31.3 — 40.8)	1.58 (0.532 —	2.27 (0.862 —

				2.93)	3.92)
Nalbuphine	6.65 (4.30 — 9.02)	110 (92.4 — 137)	ND*** [≤2.20% (5.09%) @ 178 mg/kg]	16.5 (10.2 — 31.9)	Not Applicable
SNC 80	ND*** [≤23.0% (11.1%) @ 100 mg/kg]	ND*** [≤58.3% (11.9%) @ 100 mg/kg]	ND*** [≤2.19% (3.71%) @ 100 mg/kg]	Not Applicable	Not Applicable
U69,593	ND*[≤22.4% (14.2%) @ 1.78 mg/kg]	2.49 (2.07 — 2.95)	5.73 (5.07 — 6.82)	Not Applicable	Not Applicable

Table 7 Potency ratios of morphine and mitragynine alone in the presence of various compounds to produce discriminative-stimulus, rate-decreasing, and antinociceptive effects in either morphine- or mitragynine-trained rats. Each potency ratio (95% CIs) is a combination of females and males unless otherwise noted. The ED_{50} values of morphine and mitragynine alone are shown in Table 4. The sample sizes are described in each figure legend (Figures 7—10). Significant differences are bold.

Rats Trained with Morphine			
Test compound	Discriminative Stimulus	Response Rate	Antinociception
0.032 mg/kg Naltrexone + Morphine versus Morphine Alone	First: **8.22 (5.77 — 11.6)** Reassessment: **9.50 (6.65 — 13.8)**	First: 2.58 (1.68 — 4.45) Reassessment: 1.66 (1.08 — 2.81)	Not Applicable
0.032 mg/kg Naltrexone + 7-Hydroxymitragynine versus 7-Hydroxymitragynine Alone	2.44 (1.46 — 9.69)	1.29 (0.753 — 2.32)	Not Applicable
1.78 mg/kg Nalbuphine + Morphine versus Morphine Alone	First: **0.181 (0.0918 — 0.288)** Reassessment: **0.209 (0.106 — 0.342)**	First: 1.90 (1.33 — 2.86) Reassessment: 1.22 (0.855 — 1.80)	Not Applicable
5.6 mg/kg Mitragynine + Morphine versus Morphine Alone	First: **0.258 (0.120 — 0.327)** Reassessment: **0.299 (0.139 — 0.387)**	First: 0.854 (0.555 — 1.34) Reassessment: 0.549 (0.356 — 0.845)	Not Applicable
Rats Trained with Mitragynine			
0.032 mg/kg Naltrexone + Mitragynine versus Mitragynine Alone	Not Applicable	Not Applicable	Not Applicable

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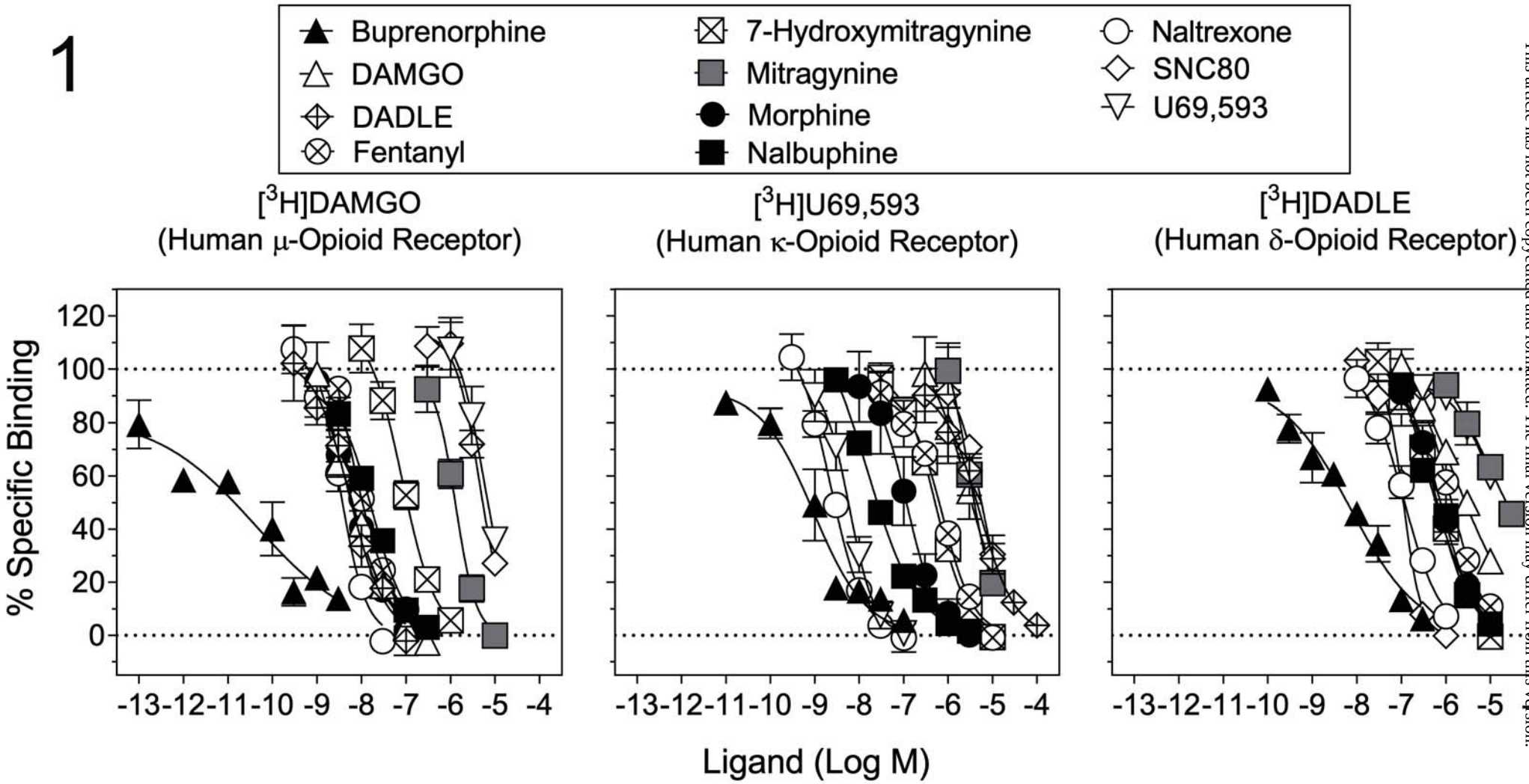

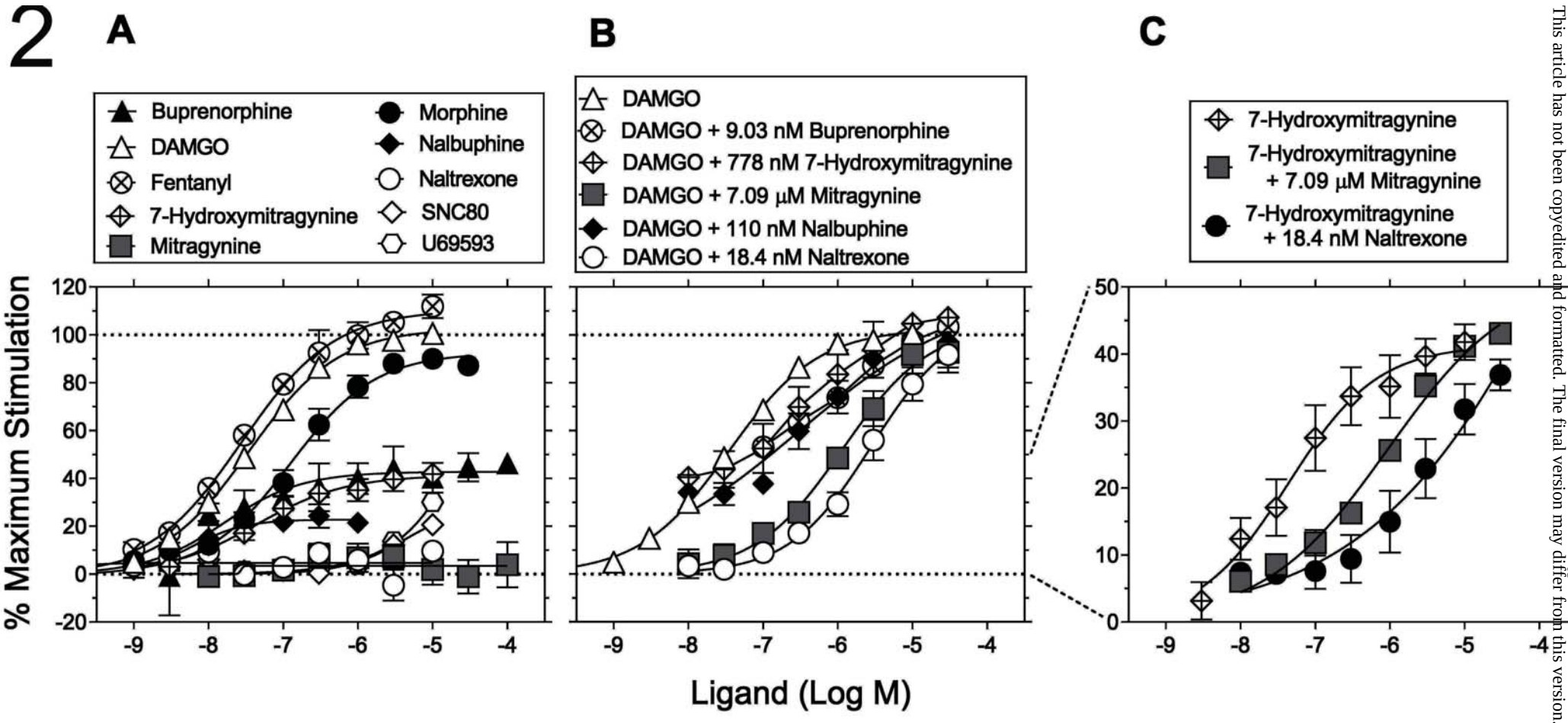

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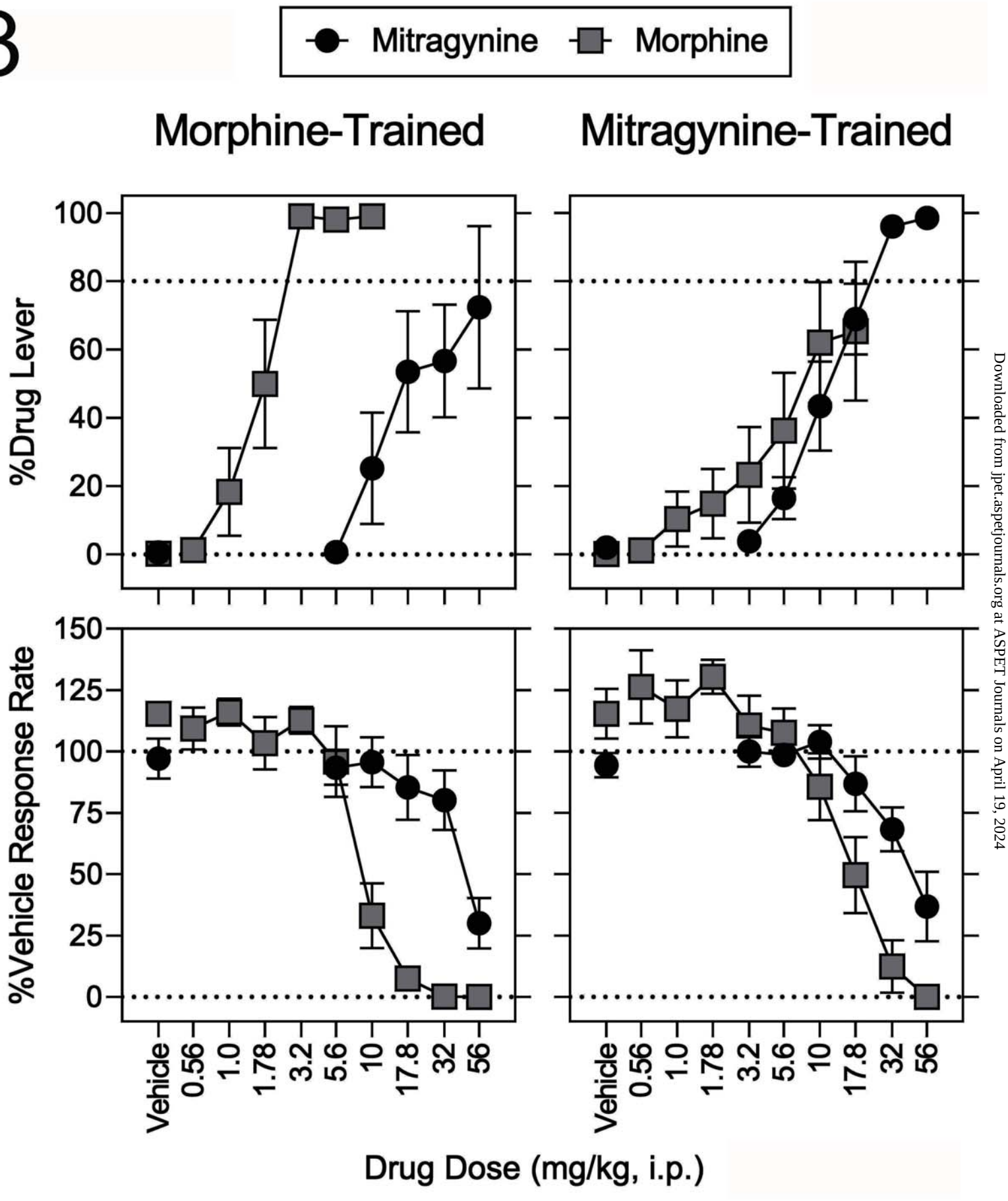

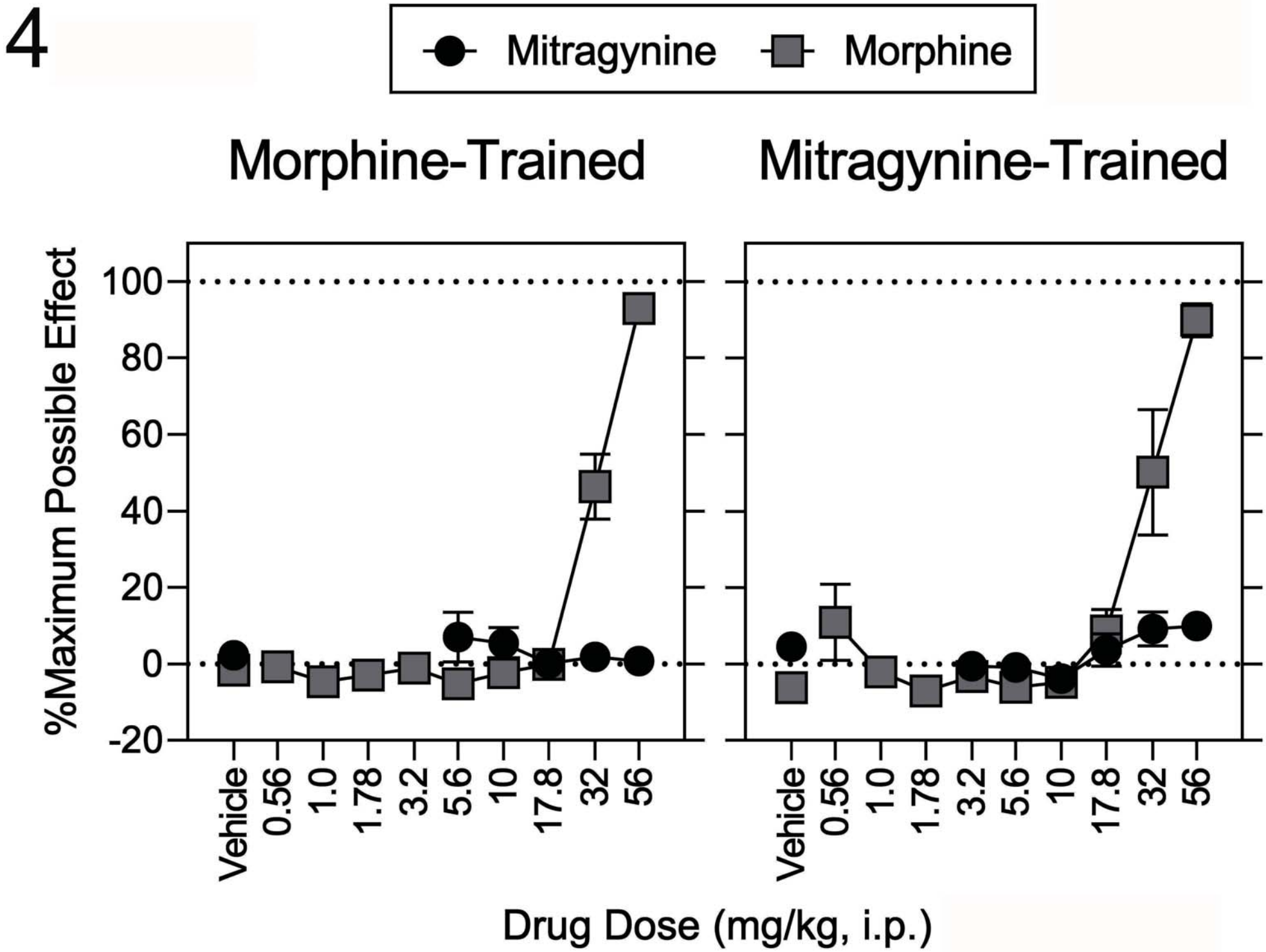

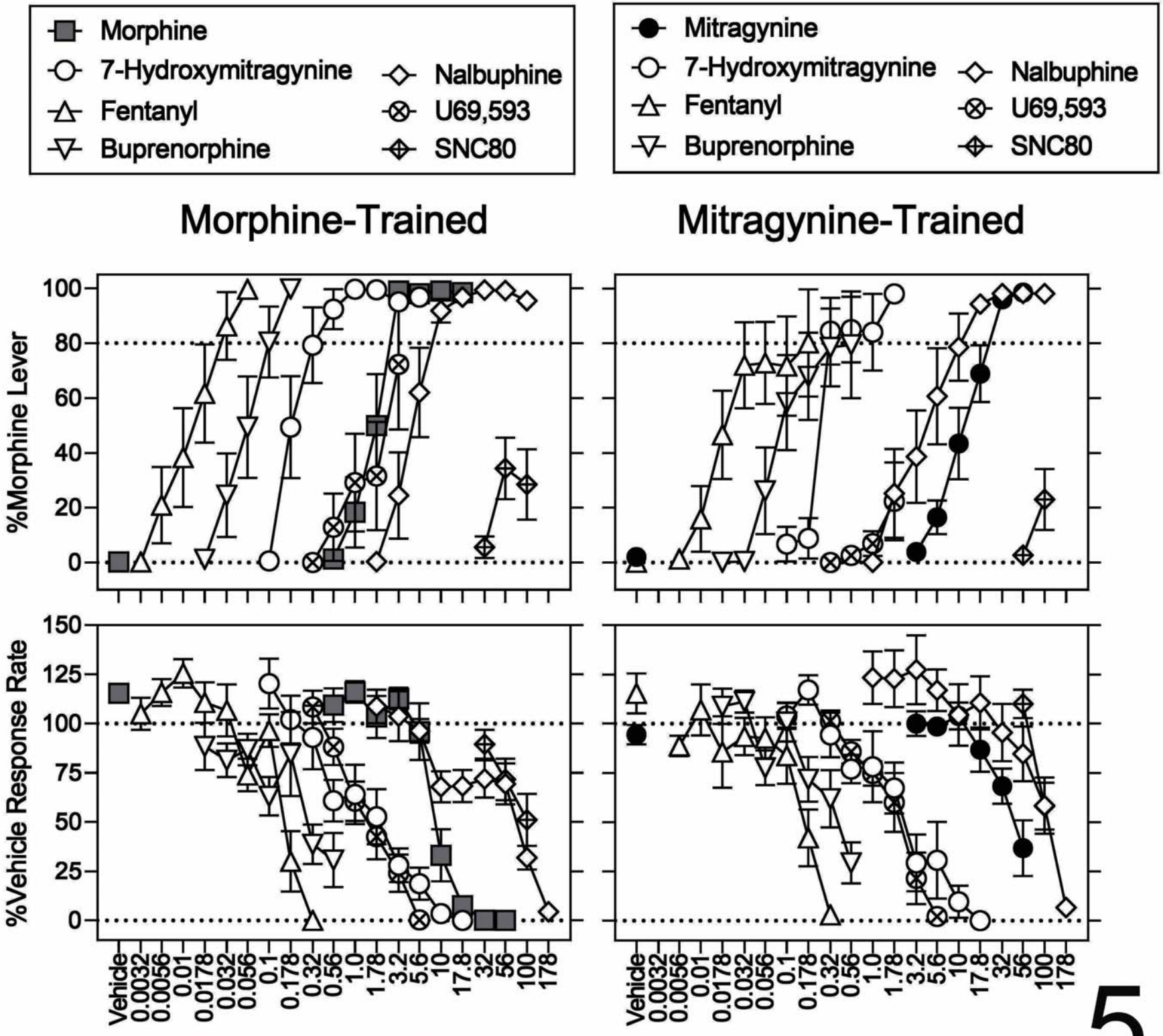

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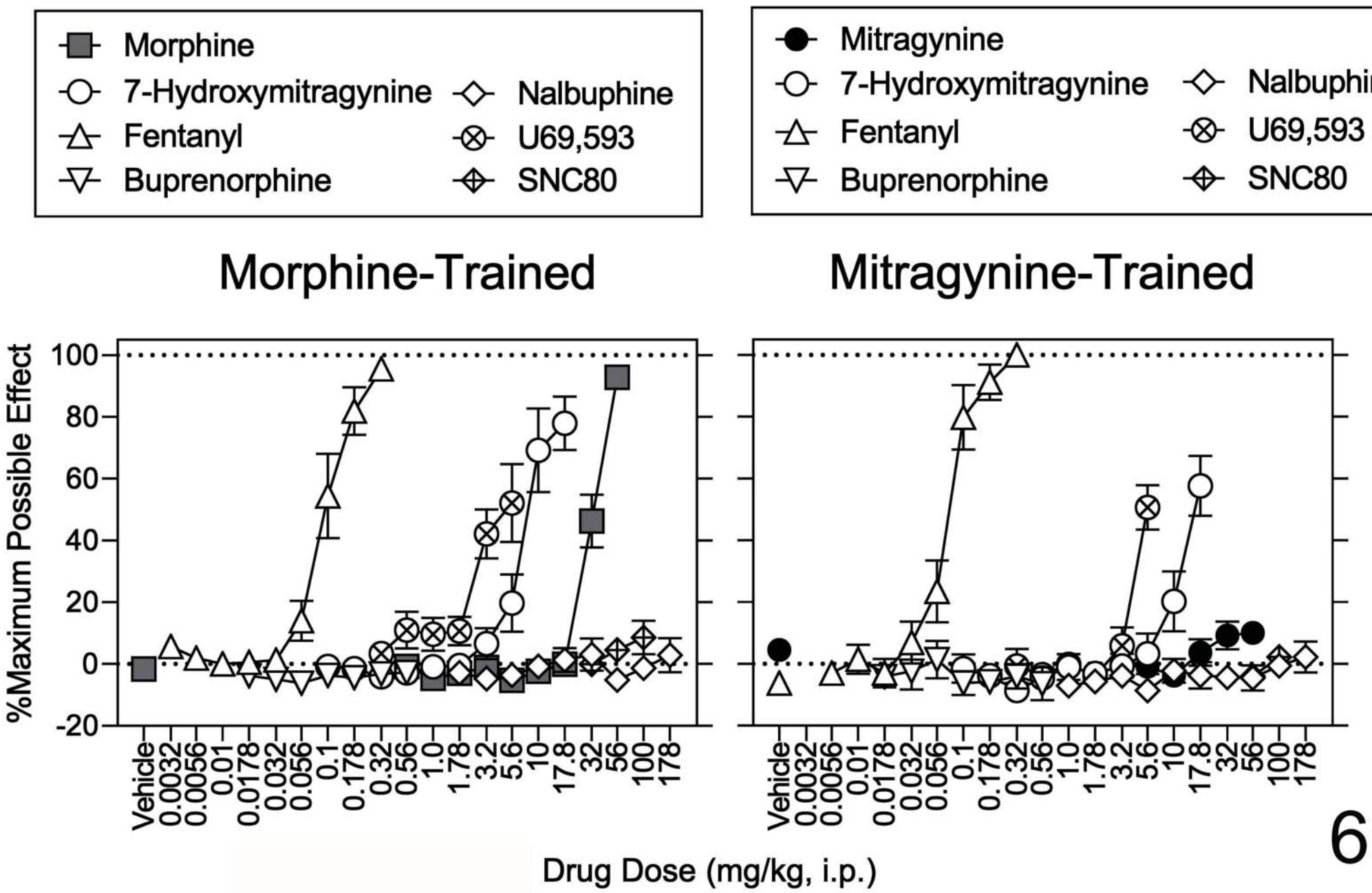

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