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Pharmacological Comparison of Mitragynine and 7-Hydroxymitragynine: In Vitro Affinity and Efficacy for μ-Opioid Receptor and Opioid-Like Behavioral Effects in Rats[S]

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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-opioid receptor (KOR) or δ-opioid receptor (DOR). [^{35}S]guanosine 5′-*O*-[γ-thio]triphosphate stimulation at MOR demonstrated that mitragynine was an antagonist, whereas 7-hydroxymitragynine was a partial agonist (E_{max} = 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 μ-opioid receptor (MOR) in vitro, mitragynine has low affinity and is an antagonist, whereas 7-hydroxymitragynine has 9-fold higher affinity than mitragynine and is an 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 nonopioid receptors.

Introduction

Opioid overdose, a leading cause of death for people under age 50 in the Unites States, has resulted in decreased life expectancy (Crimmins and Zhang, 2019; Melton and Melton, 2019). Current Food and Drug Administration–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

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ABBREVIATIONS: CI, confidence interval; DADLE, [D-Ala^2, D-Leu^5]-enkephalin; DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin; DOR, δ-opioid receptor; Emax, maximum effect achievable; FR, fixed ratio; GTPγS, guanosine 5′-*O*-[γ-thio]triphosphate; HEK, human embryonic kidney; hMOR, human MOR; IBNtxA, 3-iodobenzoyl naltrexamine; KOR, κ-opioid receptor; LED, light-emitting diode; MOR, μ-opioid receptor; MPE, maximum possible effect; TME, Tris MGCl2 EGTA.

more effective medications to achieve higher levels of abstinence than those associated with the Food and Drug Administration–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 because of 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-opioid receptor (DOR) or κ-opioid receptor (KOR) using a guanosine 5′-*O*-[γ-thio]triphosphate (GTPγS) functional assay (Váradi 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 hot-plate test (Macko et al., 1972). The antinociceptive effects of mitragynine were blocked by the nonselective 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), whereas 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 (Váradi 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 hot-plate 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 [semisynthesized 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 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 because of 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 minutes prior to sessions; other compounds were administered 15 minutes 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).

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 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 used to determine and adjust the concentration of protein required for the assay (Tal et al., 1985). Ten micrograms 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 EGTA (Sigma-Aldrich), pH 7.7] for 60 minutes at room

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% CIs.

Receptor	Source (Cell)	Radioligand	Radioligand Concentration (nM), (Mean ± S.E.M.)	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)

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 β 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 micrograms of hMOR-CHO membrane protein was incubated with 10 μM GDP, 0.1 nM [^{35}S]GTPγS, and varying concentrations of the compound under investigation for 1.5 hours 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 was used to increase agonist-stimulated binding; the final volume in each well was 300 μl. Ten micromolars 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 β 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 3 days to a temperature- (21.9 ± 1.9°C) and humidity-controlled (53% ± 14%) vivarium with a 12-hour light/dark cycle (lights on at 0700 hours). 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 minutes after 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 at the University of Florida, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and were written in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were conducted in the light cycle at the same time each day 7 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 ± 0.1°C for at least 30 minutes 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 that were 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 licking, and paw shaking; the maximum latency was 60 seconds. Immediately after a response or 60 seconds, whichever occurred first, the animal was removed from the apparatus. After the measurement of hot-plate baseline latency, each subject received an intraperitoneal injection and was returned to their home cage. Hot-plate response latency was measured a second time immediately after 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 minutes. 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 seconds [fixed-ratio (FR) 1 schedule] followed by a 0.1-second timeout during which the LEDs were turned off, the house light was illuminated, and responding had no scheduled consequences; the retractable levers remained present during the timeout. The correct lever (left vs. right) was alternated daily, and the ratio value was systematically increased each session. After 50 reinforcers per session were delivered within 20 minutes 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 minutes prior to sessions) and a second group trained to discriminate mitragynine (32 mg/kg, i.p., administered 30 minutes prior to sessions). Immediately after 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 was 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 presession 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 minutes 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 nonsystematically 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, and they were followed in a nonsystematic 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 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 because of limitations in solubility. The following drugs were administered in doses increasing by 0.25 log unit 15 minutes 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 minutes 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 (±S.E.M.) 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, intertest session, or assessment order (first vs. 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 × sex interactions were reported and further assessed with post hoc tests; significant differences are noted by asterisks on the abscissae of figures.

The hot-plate latencies were normalized to the percentage of the maximum possible antinociceptive effect (MPE) using the following formula: %MPE = 100 × (postinjection latency − preinjection baseline latency)/(maximum latency 60 seconds − preinjection 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 seconds. 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 with those of reference opioid receptor ligands (Fig. 1). The results obtained here were also compared with published values (Table 2). The δ- and κ-opioid receptor agonists, SNC 80 and U69,593, respectively, were not tested beyond 10 μM because of solubility. DADLE and SNC 80 were more potent to displace bound [^{3}H]DADLE than [^{3}H]U69,593 and [^{3}H]DAMGO, whereas U69,593 and DAMGO 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 = 2760 (1190–6930) nM] (Table 2).

Mitragynine displaced bound [^{3}H]DAMGO, [^{3}H]U69,593, and [^{3}H]DADLE in a concentration-dependent manner (Fig. 1). The binding affinity of mitragynine at MOR [K_i value = 709 (451–1130) nM] was at least 89-fold higher 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 = 1700 (1090–2710) nM] and δ-opioid [K_i value = 6800 (2980–15,900) 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 (Fig. 1). The binding affinity of 7-hydroxymitragynine at

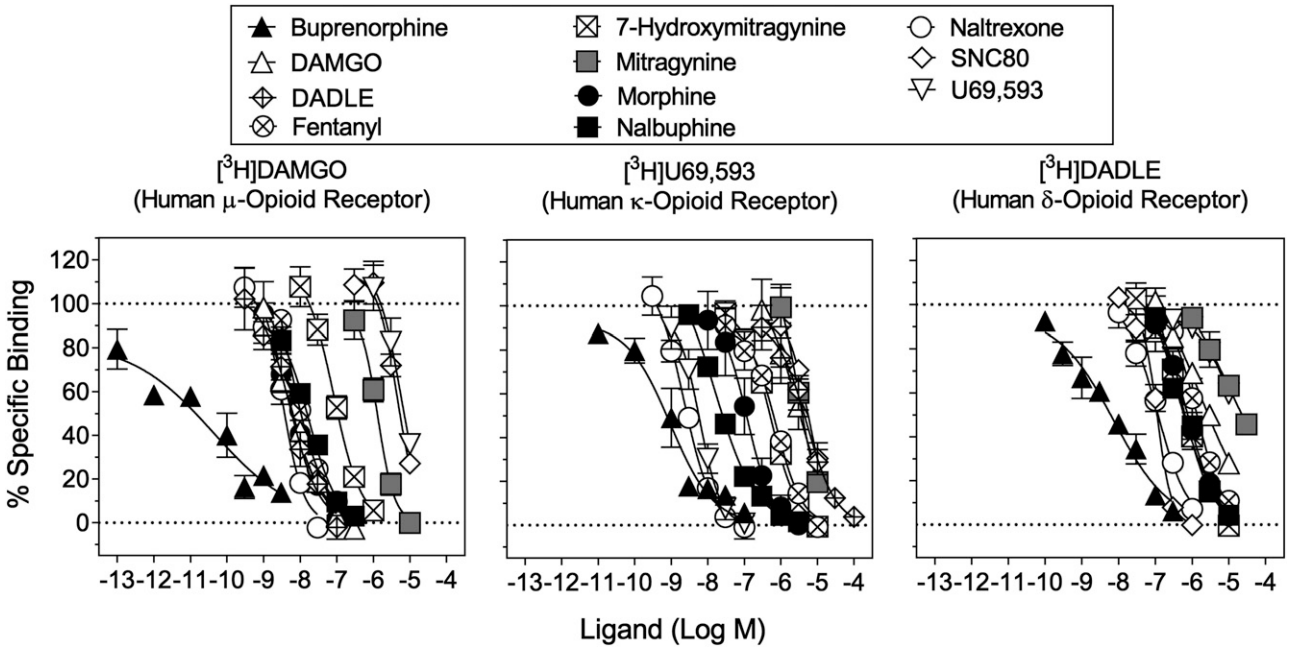

Fig. 1. Displacement of radioligands for opioid receptor subtypes. Ordinates: percentage of specific radiotracer bound to membrane preparations. Abscissae: concentrations of each competing compound (log scale). Left: displacement of [^{3}H]DAMGO labeling MORs. Middle: displacement of [^{3}H]U69,593 labeling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

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-opioid receptor than at κ- [K_i value = 220 (162–302) nM] and δ-opioid receptors [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 with those of DAMGO; fentanyl; morphine; buprenorphine; nalbuphine; naltrexone; U69,593; and SNC 80 (Fig. 2A). DAMGO, fentanyl, and

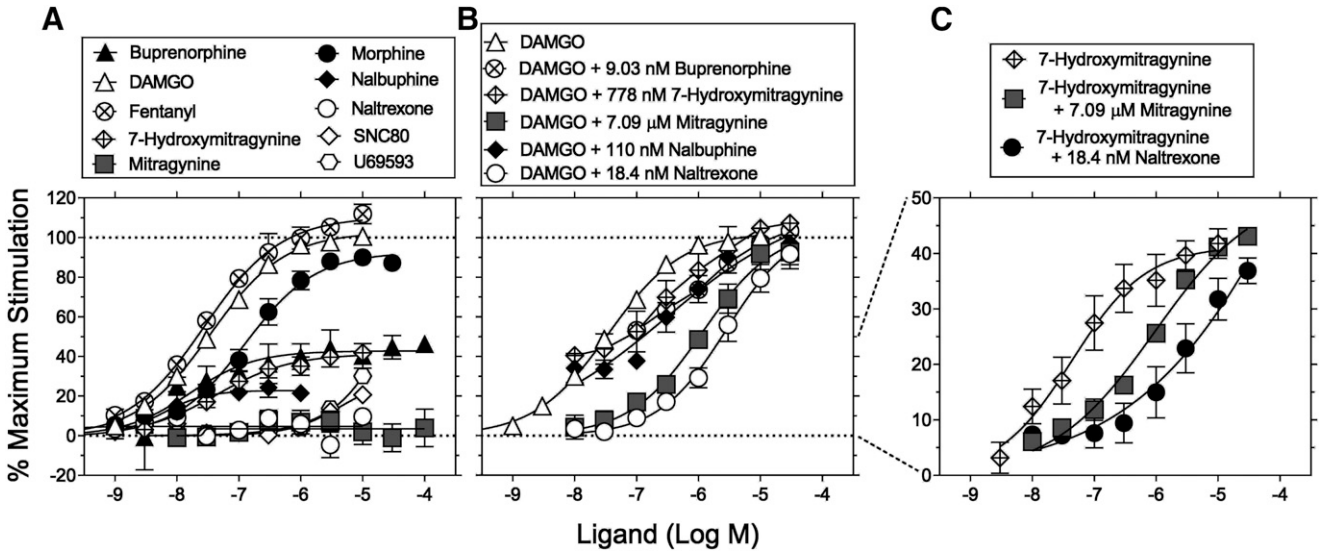

Fig. 2. [^{35}S]GTPγS stimulation in CHO cell lines stably expressing the hMORs. Ordinates: percentage of maximum stimulation of [^{35}S]GTPγS binding normalized to maximum DAMGO response as 100%. Abscissae: concentrations of each test compound (log scale). (A) Effects of test compounds alone. (B) Effects of DAMGO in combination with buffer, 110 nM ($10 \times K_i$ value at hMOR) nalbuphine, 18.4 nM ($10 \times K_i$ value at hMOR) naltrexone, 7.09 μM ($10 \times K_i$ value at hMOR) mitragynine, 779 nM ($10 \times K_i$ value at hMOR) 7-hydroxymitragynine, and 9.03 nM ($10 \times K_i$ value at hMOR) buprenorphine. (C) Effects of 7-hydroxymitragynine in the presence of buffer, 18.4 nM ($10 \times K_i$ value at hMOR) naltrexone, and 7.09 μM ($10 \times K_i$ value at hMOR) mitragynine. Data are percentages of the mean ± S.E.M. ($N \geq 3$ 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.

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 Fig. 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 (442–1930)	1200 (556–2770)	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)	243 (168–355)	220 (162–302)	2.82	3.12	1.15
	37 (S.E.M.: 4, mouse)[b]	91 (S.E.M.: 8, mouse)[b]	132 (S.E.M.: 7, mouse)[b]	3.57 (mouse)[b]	2.46 (mouse)[b]	0.69 (mouse)[b]
	47 (S.E.M.: 18, human)[c]	219 (S.E.M.: 41, human)[c]	188 (S.E.M.: 38, human)[c]	4 (human)[c]	4.66 (human)[c]	1.16 (human)[c]
	7.16 (S.E.M.: 0.94, human)[d]	236 (S.E.M.: 6, human)[d]	74.1 (S.E.M.: 7.8, human)[d]	10.4 (human)[d]	33.0 (human)[d]	3.2 (human)[d]
	70 (human)[e]	470 (human)[e]	320 (human)[e]	4.57 (human)[e]	6.71 (human)[e]	1.47 (human)[e]
Mitragynine	709 (451–1130)	6800 (2980–15,900)	1700 (1090–2710)	2.40	9.60	4.00
	230 (S.E.M.: 47, mouse)[b]	1010 (S.E.M.: 50, mouse)[b]	231 (S.E.M.: 21, mouse)[b]	1.00 (mouse)[b]	4.39 (mouse)[b]	4.37 (mouse)[b]
	233 (S.E.M.: 48, human)[c]	>10,000 (human)[c]	772 (S.E.M.: 207, human)[c]	3.31[c] (mouse)	Not determined (human)[c]	Not determined (human)[c]
	502 (S.E.M.: 19.4, rat)[f]	7910 (S.E.M.: 1,140, rat)[f]	1200 (S.E.M.: 79.7, rat)[f]	2.39 (human)[c]	15.8 (rat)[c]	6.59 (rat)[c]
	7.24 (S.E.M.: 3.44, guinea pig)[g]	60.3 (S.E.M.: 23.1, guinea pig)[g]	1100 (S.E.M.: 436, guinea pig)[g]	152 (guinea pig)[f]	8.33 (guinea pig)[f]	0.0548 (guinea pig)[f]
	740 (human)[e]	6500 (human)[e]	1300 (human)[e]	1.76 (human)[e]	8.78 (human)[e]	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	2760 (1190–6930)	34.6 (26.5–45.5)	2020 (1050–3950)	0.73	0.013	0.018
U69,593	3180 (1050–11,600)	6700 (2160–28,000)	1.62[a] (1.02–2.64)	0.0005	2.11	4140

[a] K_d 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 (Váradi 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 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).

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 (Fig. 2A). Buprenorphine and nalbuphine were partial agonists [i.e., % maximum stimulation of 42.8% and 22.8%, respectively (Fig. 2A)]. Naltrexone produced no agonism up to 10 μM (Fig. 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% stimulation of MOR at 10 μM, respectively. Higher concentrations were not tested because of solubility limitations. Mitragynine did not produce significant agonism up to 100 μM (Fig. 2A). 7-Hydroxymitragynine was a partial agonist [i.e., % maximum stimulation of 41.3% (Fig. 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-opioid receptors and up to 200 μM at δ-opioid receptor (Supplemental Fig. 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 (Fig. 2, B and C). 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 (Fig. 2B; Table 4). Mitragynine at 10× its MOR K_i value (7.09 μM, Table 2) produced 33-fold rightward shift in the concentration-effect curve of DAMGO (Fig. 2B). Buprenorphine, 7-hydroxymitragynine, and nalbuphine at 10× their MOR K_i values antagonized DAMGO 10-, 7-, and 8-fold, respectively (Fig. 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 (Fig. 2C; Table 4). The antagonist effects of mitragynine at the human δ-, κ-, and μ-opioid receptors were further tested at Eurofins Cerep 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 Figs. 2–4).

Control Performance. Baseline hot-plate response latency determined in animals discriminating either morphine [mean 8.0 (range 6.8–8.8) seconds] or mitragynine [9.6 (7.4–17.3) seconds] 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 × sex interaction (P values ≥ 0.306). Mean (S.E.M.) response rates (responses/second) were 1.0 (0.06) in morphine-trained rats and 0.90 (0.09) in 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 Supplemental Table 3 and Tables 5 and 6. In female and male rats discriminating morphine (3.2 mg/kg), vehicle produced a mean (S.E.M.) of 0.25% (0.12%) and 0.15% (0.15%) morphine-appropriate responding, respectively; mean (S.E.M.) response rates normalized to vehicle control were 120% (8.1%) and 110% (1.1%); and hot-plate response latencies expressed as MPE were −4.0% (2.4%) and 0.76% (5.0%), respectively (Supplemental Figs. 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 Figs. 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 (Supplemental Table 3; Tables 5 and 6). 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 (Supplemental Table 3; Tables 5 and 6).

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 Fig. 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)	41.3 (37.1–45.6)
	53 (S.E.M.: 4)[a]	77 (S.E.M.: 5)[a]
	7.65 (S.E.M.: 0.884)[b]	96.8 (S.E.M.: 1.8)[b]
Mitragynine	Not determined in the present study	3.46 ((−0.047 to 6.97)
	203 (S.E.M.: 13)[a]	65 (S.E.M.: 2.8)[a]
	320 (S.E.M.: 14.7)[b]	44.1 (S.E.M.: 0.62)[b]
Morphine	125 (84.8–184)	92.6 (85.8–99.4)
Nalbuphine	5.87 (4.18–8.23)	22.8 (21.1–24.6)
	9.0 (S.E.M.: 1.6)[c]	16 (S.E.M.: 0.4)[c]
	1.86 (SE: 0.1)[d]	12[d]
Naltrexone	Not determined in the present study	4.69 (0.162–9.22)
SNC 80	Not determined	20.7 (S.E.M.: 3.01) at 10 μM
U69,593	Not determined	30.1 (S.E.M.: 2.88) at 10 μM

[a][^{35}S]GTPγS functional assay in transfected CHO cells stably expressing cloned mouse MORs (Váradi et al., 2016).
[b]Homogeneous time-resolved fluorescence 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 Fig. 2.

Compound	EC_{50} (95% CI) nM	E_{max} [%DAMGO] (95% CI)	Potency Ratio (vs. DAMGO or 7-Hydroxymitragynine)
DAMGO	34.8 (24.9–48.6)	103 (96.4–109)	
DAMGO + naltrexone	2820 (1650–4820)	107 (90.7–123)	81.0 (34.0–194)
DAMGO + mitragynine	1160 (656–2050)	105 (90.0–119)	33.3 (13.5–82.3)
DAMGO + nalbuphine	289 (16.6–5030)	118 (66.3–171)	8.30 (0.34–202)
DAMGO + 7-hydroxymitragynine	235 (27.1–2030)	134 (94.2–173)	6.75 (0.56–81.5)
DAMGO + buprenorphine	360 (52.9–2450)	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	3010 (933–97,30)	46.1 (32.4–59.8)	69.4 (12.6–381)
7-Hydroxymitragynine + mitragynine	960 (287–3180)	51.9 (39.6–64.1)	22.1 (3.89–125)

In female and male rats discriminating mitragynine (32 mg/kg), mean (S.E.M.) drug-lever responding after vehicle was 2.0% (1.3%) and 2.0% (0.93%), respectively; mean (S.E.M.) response rates normalized to vehicle control were 98% (9.4%) and 91% (4.8%), respectively; and mean (S.E.M.) hot-plate response latencies expressed as MPE were 4.1% (5.8%) and 5.1% (3.8%), respectively (Supplemental Figs. 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 hot-plate assay (Supplemental Figs. 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 (Supplemental Table 3; Tables 5 and 6). Corresponding values to decrease response rates were 36 (30–46) and 65 (43–575) mg/kg, respectively (Supplemental Table 3; Tables 5 and 6). The potencies of morphine and mitragynine to produce discriminative-stimulus, rate-decreasing, and antinociceptive effects did not differ significantly between the first and second determinations (Supplemental Table 3; Tables 5 and 6).

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 ≤ 3.37; P values ≥ 0.116) and mitragynine (F values ≤ 2.03; P values ≥ 0.205, Supplemental Table 2).

Cross-Substitution. In rats discriminating morphine, 56 mg/kg of mitragynine produced a maximum of 72% (S.E.M.: 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 (Figs. 3 and 4, left, filled circles). The mitragynine ED_{50} values (95% CIs) to increase

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 Figs. 3–10 and Supplemental Figs. 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.

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%) at 32 mg/kg][a]	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%) at 0.56 mg/kg][a]	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%) at 5.6 mg/kg][a]	1.54 (0.651–3.41)	Not applicable
Morphine	1.85 (1.54–2.20)[b], 1.60 (1.30–1.91)[c]	13.7 (10.6–16.8)[b], 21.3 (16.8–26.2)[c]	35.2 (32.6–38.1)[b], 33.4 (30.9–36.4)[c]	7.41 (4.82–10.9)[b], 13.3 (8.80–20.2)[c]	19.0 (21.2–24.7)[b], 20.9 (16.2–28.0)[c]
Morphine + 0.032 mg/kg naltrexone	15.2 (12.7–17.9)	35.4 (28.2–47.2)	ND [≤3.35% (1.92%) at 56 mg/kg][d]	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%) at 56 mg/kg][d]	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%) at 56 mg/kg][d]	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%) at 178 mg/kg][e]	11.2 (6.26–41.5)	Not applicable
SNC 80	ND [≤34.3% (11.3%) at 56 mg/kg][e]	ND [≤51.2% (13.2%) at 100 mg/kg][e]	ND [≤8.63% (5.47%) at 100 mg/kg][e]	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)

[a]Due to lethality.
[b]First assessment.
[c]Reassessment.
[d]Due to an adverse reaction (scratching behavior).
[e]Due to insolubility in the chosen vehicle.

TABLE 6

ED_{50} values (95% CIs) for the discriminative-stimulus, rate-decreasing, and antinociceptive effects of various compounds in rats trained to discriminate 32 mg/kg mitragynine as shown in Figs. 3–10 and Supplemental Figs. 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.

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%) at 0.056 mg/kg][a]	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)[b], 12.8 (9.31–16.0)[c]	46.2 (38.1–60.6)[b], 47.2 (38.2–65.5)[c]	ND [up to 10.0% (3.32%)[b] and 8.01% (4.80%) at 5.6 mg/kg[c]][d]	3.06 (2.16–4.77)[b], 3.69 (2.39–7.04)[c]	Not applicable
Mitragynine + 0.032 mg/kg naltrexone	ND [≤27.2% (17.1%) at 56 mg/kg][d]	ND [≤53.2% (10.8%) at 56 mg/kg][d]	ND [≤ −3.91% (4.56%) at 56 mg/kg][d]	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 (0.532–2.93)	2.27 (0.862–3.92)
Nalbuphine	6.65 (4.30–9.02)	110 (92.4–137)	ND [≤2.20% (5.09%) at 178 mg/kg][e]	16.5 (10.2–31.9)	Not applicable
SNC 80	ND [≤23.0% (11.1%) at 100 mg/kg][e]	ND [≤58.3% (11.9%) at 100 mg/kg][e]	ND [≤2.19% (3.71%) at 100 mg/kg][e]	Not applicable	Not applicable
U69,593	ND[≤22.4% (14.2%) at 1.78 mg/kg][d]	2.49 (2.07–2.95)	5.73 (5.07–6.82)	Not applicable	Not applicable

ND, Not determined.
[a]Due to an adverse reaction (skin ulcer).
[b]First assessment.
[c]Reassessment.
[d]Due to lethality.
[e]Due to insolubility in the chosen vehicle.

morphine-lever responding and to decrease response rates were 30 (19–60) and 46 (36–64) mg/kg (Supplemental Table 3; Tables 5 and 6). In rats discriminating mitragynine, there was no significant effect of sex and no morphine dose × sex interaction for the discriminative-stimulus, rate-decreasing, and antinociceptive effects (Supplemental Table 2). Morphine produced a maximum of 65% (S.E.M.: 20%) mitragynine-lever responding at 17.8 mg/kg; the 56 mg/kg dose of morphine eliminated responding and increased MPE to 90% (S.E.M.: 4.3%) (Figs. 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 (Supplemental Table 3; Tables 5 and 6). 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 × sex interaction (P = 0.002, Supplemental Table 2). Post hoc testing indicated significant differences that are shown in Supplemental Fig. 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).

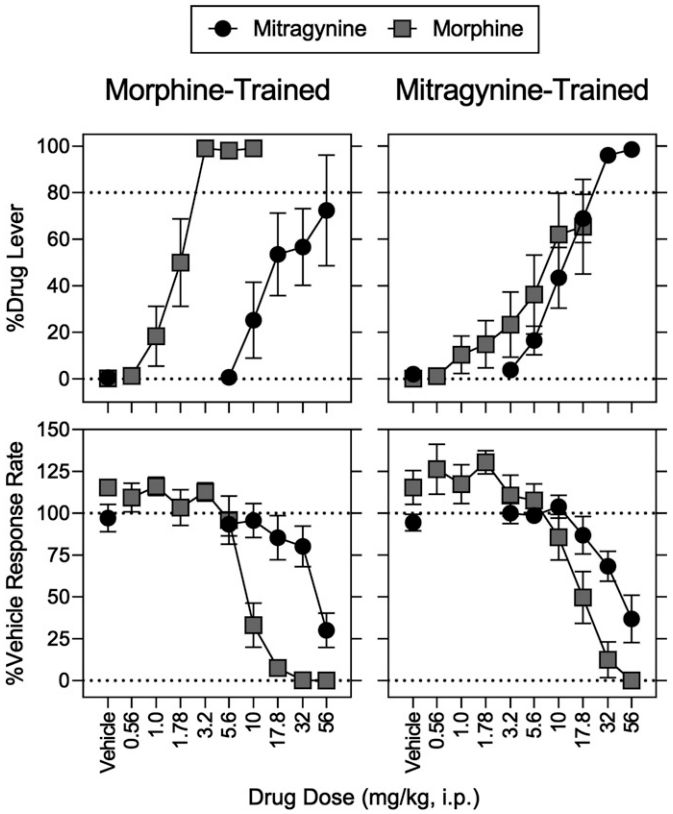

Fig. 3. Mitragynine substitution in rats trained to discriminate morphine (left) and morphine substitution in rats trained to discriminate mitragynine (right). Abscissae: vehicle and drug dose in milligrams per kilogram (intraperitoneal, log scale). Ordinates: top, percentage of responses on the training drug–appropriate lever. Bottom, mean rates of responding expressed as a percentage of vehicle control. Morphine and mitragynine were administered intraperitoneally, respectively, at 15 and 30 minutes before sessions. Each point represents the mean ± S.E.M. (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 mitragynine discrimination (N = 6) mg/kg. Details for statistical analyses are shown in Supplemental Tables 2–4 and Tables 5 and 6.

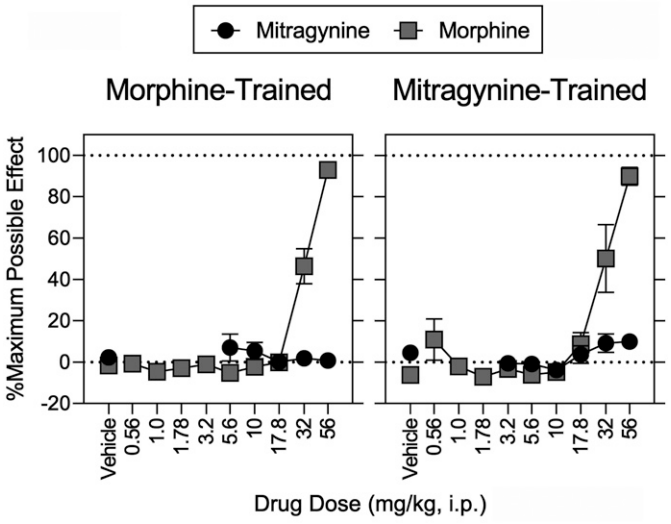

Fig. 4. Antinociceptive effects determined in conjunction with the discrimination tests shown in Fig. 5. Abscissae: vehicle and drug dose in mg/kg (intraperitoneal, log scale) in separate groups of rats discriminating either morphine (left) or mitragynine (right). Ordinates: percentage of maximum possible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

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 (Figs. 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 (Supplemental Table 3; Tables 5 and 6). 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 (Figs. 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 (Supplemental Table 3; Tables 5 and 6).

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%) (Figs. 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 (Supplemental Table 3; Tables 5 and 6). In mitragynine-trained rats, fentanyl produced a maximum of 81% (S.E.M.: 20%) drug-lever responding at 0.178 mg/kg; 0.32 mg/kg decreased responding to 3.0% of vehicle control and increased

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 (Figs. 7–10). Significant differences are italicized.

Rats Trained with Morphine			
Test Compound	Discriminative Stimulus	Response Rate	Antinociception
0.032 mg/kg Naltrexone + morphine vs. 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 vs. 7-hydroxymitragynine alone	*2.44 (1.46–9.69)*	1.29 (0.753–2.32)	Not applicable
1.78 mg/kg Nalbuphine + morphine vs. 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 vs. 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 vs. mitragynine alone	Not applicable	Not applicable	Not applicable

MPE to 100% (Figs. 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 (Supplemental Table 3; Tables 5 and 6). There was a significant fentanyl dose × sex interaction on mitragynine-appropriate responding (Supplemental Table 2). Post hoc testing suggested that fentanyl was more potent in females than males (Supplemental Fig. 6; Supplemental Table 2). 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%) (Figs. 5 and 6, left, crosshatch 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 (Supplemental Table 3; Tables 5 and 6). There was a significant U69,593 dose × 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% (S.E.M.: 14%) drug-lever responding at 1.78 mg/kg [40% (S.E.M.: 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 (Figs. 7 and 8, right, circles with cross hatch). The ED_{50} values 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 (Supplemental Table 3; Tables 5 and 6). 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%) (Figs. 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% (S.E.M.: 11%) drug-lever responding; 100 mg/kg decreased response rates to 58% (12%) of control and did not significantly increase MPE (Figs. 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 (Figs. 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 (Supplemental Table 3; Tables 5 and 6). In mitragynine-trained rats, the 0.56 mg/kg dose of buprenorphine produced a maximum of 79% (S.E.M.: 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 (Figs. 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 (Supplemental 3; Tables 5 and 6). Buprenorphine did not show any antinociceptive activity here; however, the antinociceptive effects of

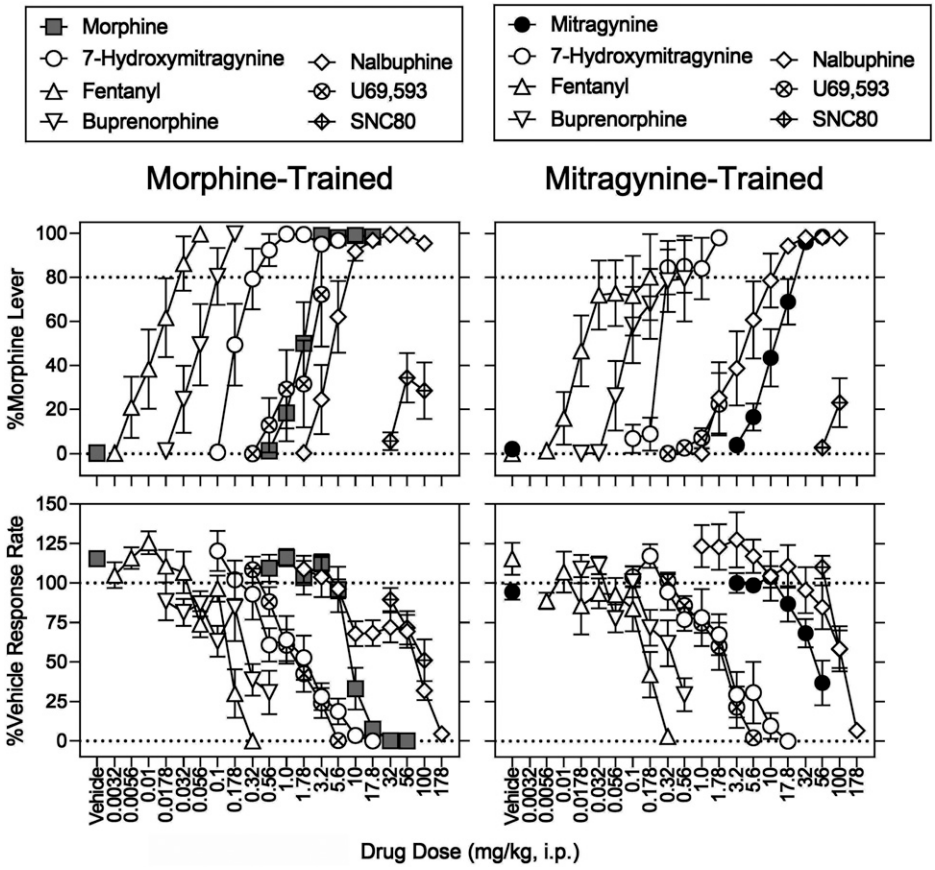

Fig. 5. Substitution tests in separate groups of rats discriminating either morphine (left) or mitragynine (right). Abscissae: vehicle and drug dose in mg/kg (intraperitoneal, log scale). Ordinates: top, percentage of responses on the training drug-appropriate lever. Bottom, mean rates of responding expressed as a percentage of vehicle control. All compounds were administered intraperitoneally 15 minutes before sessions except mitragynine (30 minutes prior to sessions). The training drug dose-effect functions are replotted from Fig. 5. Each point represents the mean ± S.E.M. ($N = 8$) except in the morphine discrimination [7-hydroxymitragynine at 0.56, 1.0, and 1.78 mg/kg ($N = 7$) and 3.2 mg/kg ($N = 5$); buprenorphine at 0.178 mg/kg ($N = 5$); nalbuphine at 100 mg/kg ($N = 7$); 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 Supplemental Tables 2–4 and Tables 5 and 6.

0.32 mg/kg buprenorphine at a lower temperature (50°C) were robust (100%) in naïve female and male rats in a preliminary study (unpublished data).

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 (Figs. 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 (Supplemental Table 3; Tables 5 and 6). In mitragynine-trained rats, nalbuphine produced a maximum of 98% (S.E.M.: 0.9%) drug-lever responding at 56 mg/kg; 178 mg/kg markedly decreased rates and produced a 2.2% (5.1%) MPE (Figs. 5 and 6, right, open diamonds). The ED_{50} values of nalbuphine to increase mitragynine-lever responding and to decrease response rates

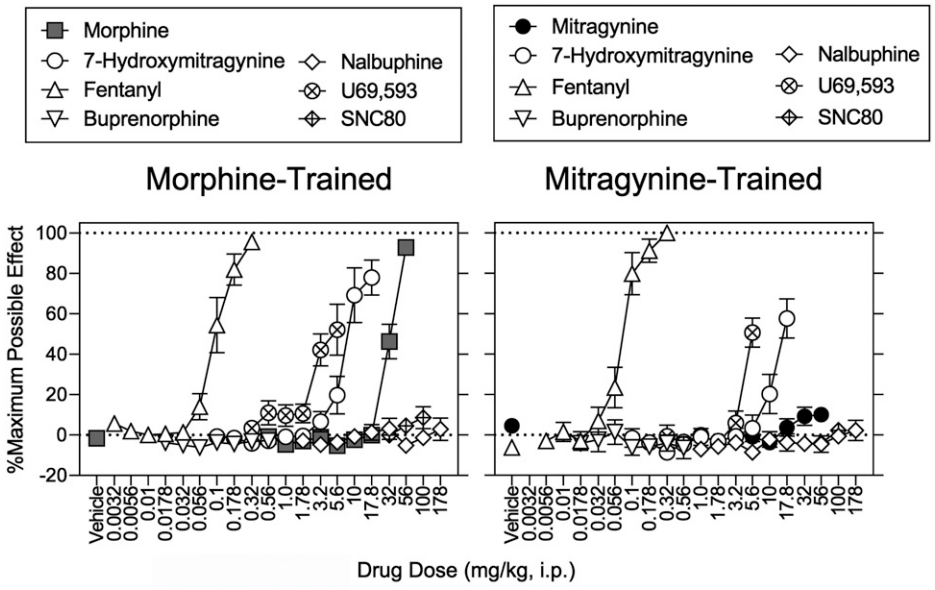

Fig. 6. Antinociceptive effects determined in conjunction with the discrimination tests shown in Fig. 7. Abscissae: vehicle and drug dose in mg/kg (intraperitoneal, log scale). Ordinates: percentage of maximum possible antinociceptive effects. Each point represents the mean ± S.E.M. ($N = 8$). All compounds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

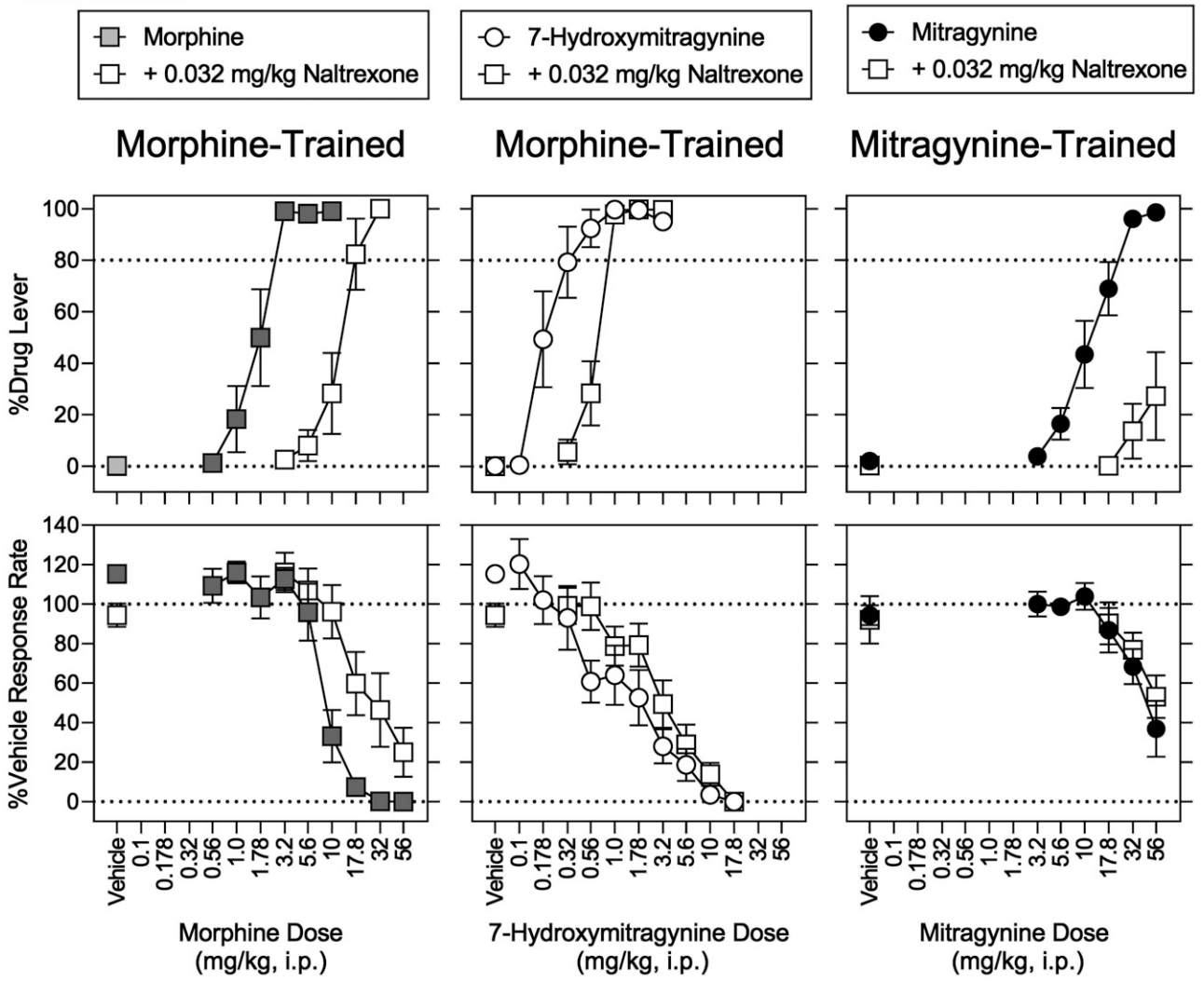

Fig. 7. Naltrexone antagonism of the effects of morphine and 7-hydroxymitragynine in rats discriminating morphine and mitragynine in rats discriminating mitragynine. Abscissae: vehicle and dose in mg/kg (intraperitoneal, log scale) for mitragynine (left), 7-hydroxymitragynine (middle), and mitragynine (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 intraperitoneally 15 minutes before sessions, and mitragynine and naltrexone (0.032 mg/kg) were administered intraperitoneally 30 minutes before sessions. The dose-effect functions for the training drugs morphine and mitragynine are replotted from Fig. 5, and the dose-effect function for mitragynine is replotted from Fig. 7. Each point represents the mean ± S.E.M. (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 Supplemental Tables 2–4 and Tables 5 and 6.

were 6.7 (4.3–9.0) and 110 (92–137) mg/kg, respectively (Supplemental Table 3; Tables 5 and 6). Nalbuphine did not significantly increase MPE (Figs. 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 (Figs. 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 redetermined (9.5-fold) (Fig. 7, top left, open squares; Table 7). 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 (Fig. 7, bottom left, open squares; Table 7). 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) × 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

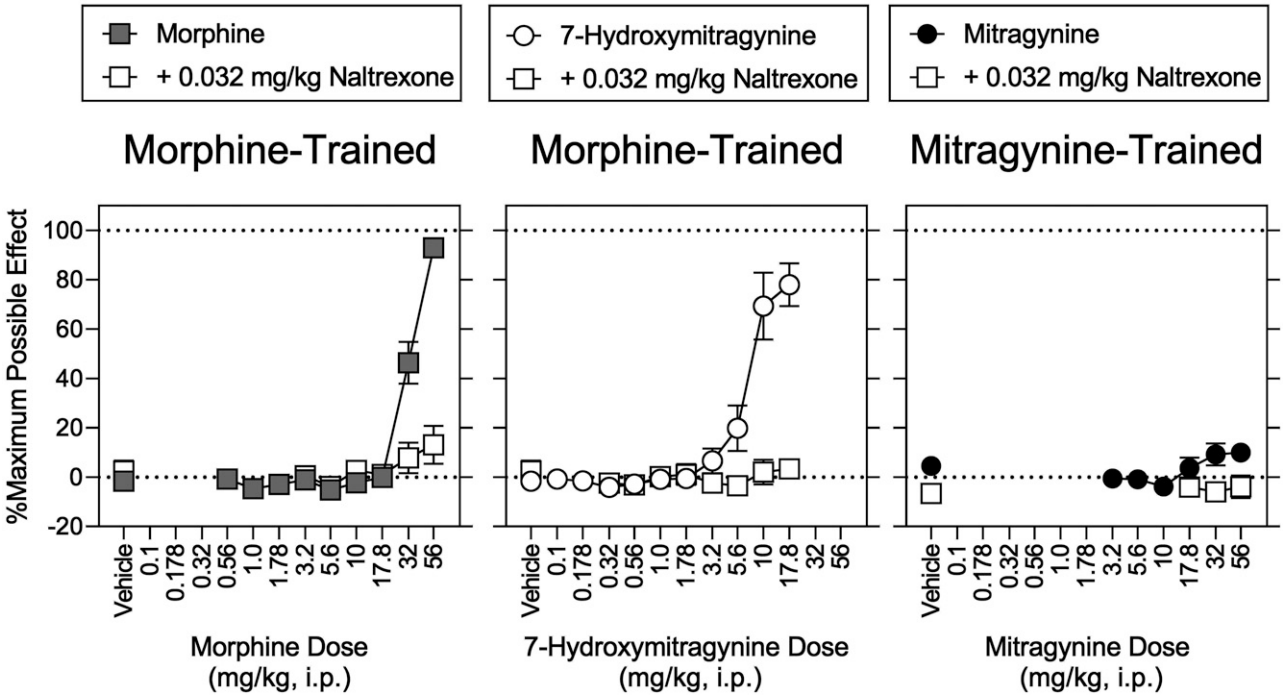

Fig. 8. Antinociceptive effects determined in conjunction with the discrimination tests shown in Fig. 9. Abscissae: vehicle and drug dose in mg/kg (intraperitoneal, log scale). Ordinates: percentage of maximum possible antinociceptive effects. Each point represents the mean ± S.E.M. (N = 8). Morphine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

morphine dose × 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 (Figs. 7 and 8, right, open squares above vehicle). In the presence of naltrexone, 56 mg/kg of mitragynine produced a maximum of 27% (S.E.M.: 17%) drug-lever responding, decreased response rates to 53% (S.E.M.: 11%) of vehicle controls, and produced −3.9% (4.6%) MPE (Figs. 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 because of 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 (Fig. 7, top middle, open squares; Table 7). Naltrexone, at 0.032 mg/kg, did not significantly antagonize the rate-decreasing effects of 7-hydroxymitragynine (Fig. 7, bottom middle, open squares; Table 7). 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%) (Fig. 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 (Figs. 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 (Fig. 9, top left, open squares; Table 7). 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 dose-effect functions were shifted rightward 1.9-fold and greater than 1.6-fold, respectively (Fig. 9, bottom left and Fig. 10, left, open squares; Table 7). 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 (Figs. 9 and 10, right, open squares above vehicle). The same dose of mitragynine significantly increased the potency of morphine 3.3-fold (Fig. 9, top right, open squares; Table 6). In contrast, and in the same animals during the same experimental sessions, mitragynine (5.6 mg/kg) antagonized the antinociceptive effects of morphine (Fig. 10, right, open squares) and did not significantly modify the morphine dose-effect function for rate-decreasing effects (Fig. 9, bottom right, open squares; Table 7).

Discussion

Pharmacological mechanisms of two kratom alkaloids, mitragynine and 7-hydroxymitragynine, were assessed 1) in cell membranes expressing hMOR and 2) in 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 an MOR antagonist, whereas

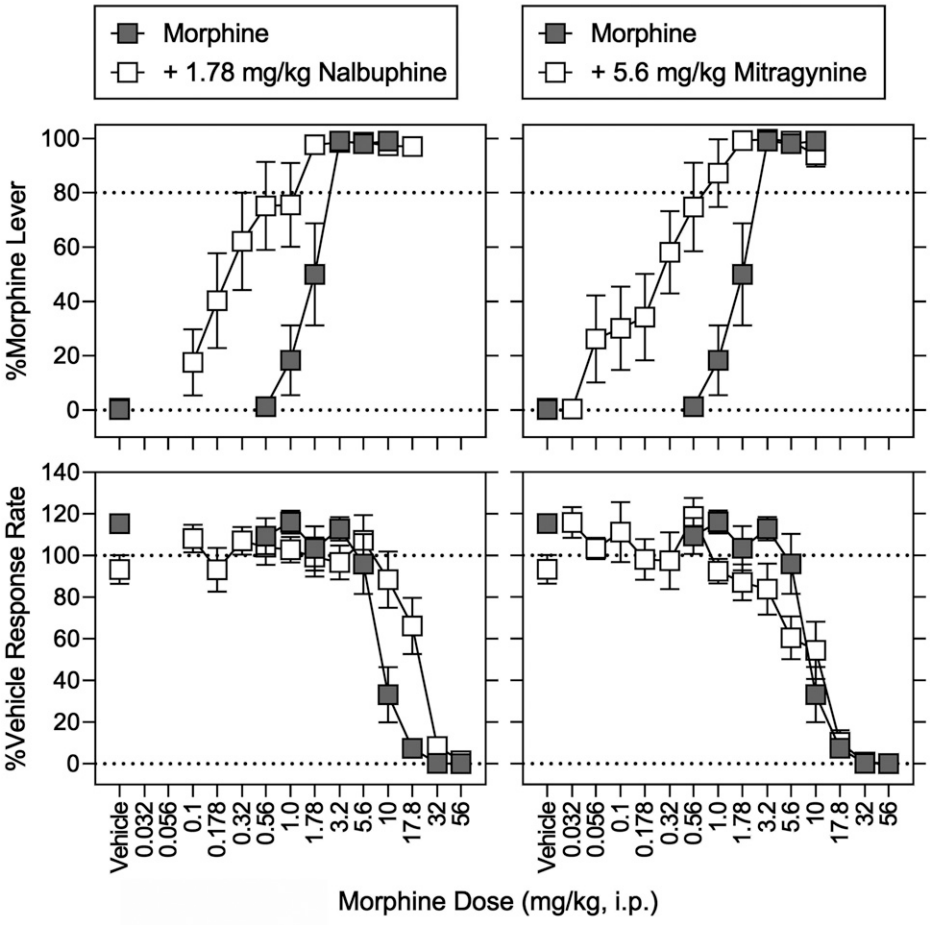

Fig. 9. Discriminative-stimulus effects of morphine in combination with nalbuphine (left) and mitragynine (right). Abscissae: vehicle and morphine dose in milligrams per kilogram (intraperitoneal, log scale). Ordinates: top, percentage of responses on the morphine-appropriate lever. Bottom, mean rates of responding expressed as a percentage of vehicle control. Morphine and nalbuphine (1.78 mg/kg) were administered intraperitoneally 15 minutes before sessions, whereas mitragynine (5.6 mg/kg) was administered intraperitoneally 30 minutes before sessions. The morphine dose-effect function is replotted from Fig. 5. Each point represents the mean ± S.E.M. (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 Supplemental Tables 2–4 and Tables 5 and 6.

7-hydroxymitragynine was an 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].

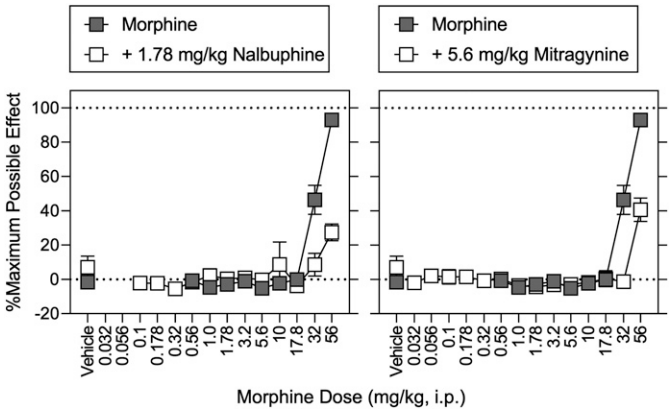

Fig. 10. Antinociceptive effects determined in conjunction with the discrimination tests shown in Fig. 9. Abscissae: vehicle and drug dose in mg/kg (intraperitoneal, log scale). Ordinates: percentage of maximum possible antinociceptive effects. Each point represents the mean ± S.E.M. (N = 8). Morphine and nalbuphine were administered intraperitoneally 15 minutes before sessions, and mitragynine was administered intraperitoneally 30 minutes before sessions. The morphine dose-effect function is replotted from Fig. 6. Details for statistical analyses are shown in Supplemental Tables 2–4 and Tables 5 and 6.

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 an MOR antagonist, and 7-hydroxymitragynine was an MOR partial agonist. Both mitragynine and 7-hydroxymitragynine were MOR partial agonists in previous studies (Kruegel et al., 2016; Váradi 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, such as hMOR (Kruegel et al., 2016; Obeng et al., 2020) versus rodent MOR (Váradi et al., 2016). The experimental techniques have differed (e.g., bioluminescence resonance energy transfer and homogeneous time-resolved fluorescence

(Kruegel et al., 2016; Váradi 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 vs. another), actions at the lower affinity site can be detected by systematically increasing the training dose (e.g., nicotine; Jutkiewicz 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 with 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 nonopioid 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,b; 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 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 nonopioid receptors in the actions of mitragynine (Boyer et al., 2008; Ellis et al., 2020; Obeng et al., 2020) as compared with nalbuphine.

An 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., 2020), and it functioned as an α_2-adrenergic agonist in mice using a hot-plate assay (Matsumoto et al., 1996a). The present results strongly suggest that mitragynine is a low-efficacy MOR agonist in vivo with additional actions at nonopioid 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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Supplemental Materials

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

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Methods

In Vitro [^{35}S]GTPγS Functional Assay at opioid receptor subtypes. The [^{35}S]GTPγS functional assay was conducted to determine the efficacy of the compounds at the opioid receptor subtypes at Eurofins Cerep (Celle l'Evescault, France). The methods are summarized in **Supplemental Table 1.** Briefly, membrane protein was incubated with guanosine diphosphate (GDP), [^{35}S]GTPγS, and varying concentrations of the compound under investigation for 30 minutes at 30°C using 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), 1.0 mM EDTA as assay buffer. Furthermore, DPDPE, U69,593, and DAMGO were included in the assay as the maximally effective concentration of the full agonist at the delta-, kappa-, and mu-opioid receptors (DOR, KOR, and MOR), respectively. The antagonist effect of mitragynine at the three opioid receptors were compared to the antagonist effect of naltrindole, nor-binaltorphimine, and naltrexone at the delta-, kappa-, and mu-opioid receptors, respectively. To test for antagonism, each agonist (unspecified from Eurofins Cerep) was incubated with increasing concentrations of the corresponding antagonists or mitragynine. After incubation, the bound radioligand was separated from the free radioligand and quantified using a liquid scintillation counter. All assays were determined in duplicate. Percent agonist-stimulated [^{35}S]GTPγS binding was defined as [(net-stimulated binding by a test compound)/(net-stimulated binding by agonist)]×100%.

Supplemental Table 1 Summary of the methods used to determine functional effects of mitragynine at the delta-, kappa-, and mu-opioid receptors from Eurofins Cerep (Celle l'Evescault, France).

Receptor	Delta-Opioid Receptor	Kappa-Opioid Receptor	Mu-Opioid Receptor
Target	Human CHO cells	*Human Chem-1 cells Rat hematopoietic cells	Human CHO-K1 cells
Vehicle	1.00% DMSO		
Incubation Time and Temperature	30 minutes @ 30°C		
Incubation Buffer	20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA		
Quantitation Method	Bound [^{35}S]GTPγS		
Replicates	2		

*This description like two cell lines is what is written in the report from Eurofins Cerep.

Supplemental Table 2 Statistical analyses of sex differences in dose-effect functions for the discriminative-stimulus, rate-decreasing, and antinociceptive effects of various compounds tested as shown in Figures 3—10 and Supplemental Figures 5—7. The sample sizes are described in each figure legend. Comparisons were made using a two-way repeated-measures ANOVA followed by *post hoc* Bonferroni *t* tests with results shown when effects were statistically significant. Significant differences are bold. #First assessment, ##Reassessment. N/A: Not applicable.

Rats Trained with Morphine				
Discriminative-Stimulus Effects				
Drug	Sex	Dose (mg/kg)	Interaction	*Post-Hoc* Test
Morphine#	$F_{1,30}$=2.66; P=0.154	$F_{5,30}$=30.9; **P<0.001**	$F_{5,30}$=1.97; P=0.112	*Sex*: 1.0 mg/kg (t=2.11, **P=0.042**), 1.78 mg/kg (t=2.89, **P=0.007**). *Dose*: 1.78 mg/kg (t=4.29, **P<0.001**), 3.2 mg/kg (t=8.52, **P<0.001**), 5.6 mg/kg (t=8.44, **P<0.001**)
Morphine##	$F_{1,34}$=0.914; P=0376	$F_{6,34}$=50.6; **P<0.001**	$F_{6,34}$=3.28; **P=0.012**	*Sex*: 1.0 mg/kg (t=4.22, **P<0.001**). *Dose*: 1.0 mg/kg (t=3.11, **P=0.023**), 1.78 mg/kg (t=8.30, **P<0.001**), 3.2 mg/kg (t=11.0, **P<0.001**), 5.6 mg/kg (t=10.9, **P<0.001**), 10 mg/kg (t=10.1, **P<0.001**)
Mitragynine Substitution for Morphine	$F_{1,26}$=0.0661; P=0.805	$F_{5,26}$=5.65; **P=0.001**	$F_{5,26}$=0.0716 ; P=0.996	*Sex*: N/A. *Dose*: 17.8 mg/kg (t=3.33, **P=0.013**), 32 mg/kg (t=3.53, **P=0.008**), 56 mg/kg (t=3.19, **P=0.018**)
Fentanyl Substitution for Morphine	$F_{1,36}$=1.43; P=0.276	$F_{6,36}$=13.8; **P<0.001**	$F_{6,36}$=0.587; P=0.739	*Sex*: N/A. *Dose*: 0.0178 mg/kg (t=4.04, **P=0.002**), 0.032 mg/kg (t=5.66, **P<0.001**), 0.056 mg/kg (t=6.54, **P<0.001**)
Buprenorphine Substitution for Morphine	$F_{1,27}$=1.51; P=0.259	$F_{5,27}$=12.2; **P<0.001**	$F_{5,27}$=1.60; =0.195	*Sex*: 0.032 mg/kg (t=2.23, **P=0.034**), 0.056 mg/kg (t=2.34, **P=0.027**). *Dose*: 0.056 mg/kg (t=3.53, **P=0.008**), 0.1 mg/kg (t=5.77, **P<0.001**), 0.178 mg/kg (t=4.74, **P<0.001**)

Nalbuphine Substitution for Morphine	$F_{1,47}$=0.0147; P=0.908	$F_{8,47}$=28.6; **P<0.001**	$F_{8,47}$=0.138; P=0.997	*Sex*: N/A. *Dose*: 5.6 mg/kg (t=5.41, **P<0.001**), 10 mg/kg (t=8.04, **P<0.001**), 17.8 mg/kg (t=8.47, **P<0.001**), 32 mg/kg (t=8.71, **P<0.001**), 56 mg/kg (t=8.69, **P<0.001**), 100 mg/kg (t=7.89, **P<0.001**)
7-Hydroxymitragynine Substitution for Morphine	$F_{1,28}$=3.12; P=0.127	$F_{5,28}$=24.2; **P<0.001**	$F_{5,28}$=1.49; P=0.225	*Sex*: 0.178 mg/kg (t=2.59, **P=0.014**), 0.32 mg/kg (t=2.16, **P=0.038**). *Dose*: 0.178 mg/kg (t=3.93, **P=0.003**), 0.32 mg/kg (t=6.33, **P<0.001**), 0.56 mg/kg (t=7.17, **P<0.001**), 1.0 mg/kg (t=7.66, **P<0.001**)
U69,593 Substitution for Morphine	$F_{1,29}$=0.743; P=0.396	$F_{5,29}$=2.98; **P=0.027**	$F_{5,29}$=0.438; P=0.818	*Sex*: N/A. *Dose*: 3.2 mg/kg (t=3.28, **P=0.013**)
SNC 80 Substitution for Morphine	$F_{1,17}$=0.0578; P=0.818	$F_{3,17}$=4.31; **P=0.020**	$F_{3,17}$=1.15; P=0.359	*Sex*: N/A. *Dose*: 56 mg/kg (t=3.00, **P=0.024**)
Morphine + 0.032 mg/kg naltrexone	$F_{1,29}$=4.19; **P=0.050**	$F_{4,29}$=13.7; **P<0.001**	$F_{4,29}$=1.11; P=0.372	*Sex*: 10.0 mg/kg (t=2.43, **P=0.021**). *Dose*: 17.8 mg/kg (t=6.36, **P<0.001**)
7-Hydroxymitragynine Substitution for Morphine + 0.032 mg/kg naltrexone	$F_{1,22}$=0.138; P=0.723	$F_{4,22}$=56.7; **P<0.001**	$F_{4,22}$=0.617; P=0.655	*Sex*: N/A. *Dose*: 0.56 mg/kg (t=3.26, **P=0.014**), 1.0 mg/kg (t=11.3, **P<0.001**), 1.78 mg/kg (t=10.2, **P<0.001**)
Morphine + 1.78 mg/kg Nalbuphine	$F_{1,59}$=2.63; P=0.156	$F_{10,59}$=14.3; **P<0.001**	$F_{10,59}$=1.89; P=0.065	*Sex*: 0.56 mg/kg (t=3.58, **P<0.001**), 1.0 mg/kg (t=2.67, **P=0.028**). *Dose*: 0.178 mg/kg (t=3.06, **P=0.034**), 0.32 mg/kg (t=4.72, **P<0.001**), 0.56 mg/kg (t=4.76, **P<0.001**), 1.0 mg/kg (t=5.75, **P<0.001**), 1.78 mg/kg (t=7.44, **P<0.001**), 3.2 mg/kg (t=7.50, **P<0.001**), 5.6 mg/kg

				(t=7.51, **P<0.001**), 10 mg/kg (t=7.41, **P<0.001**), 17.8 mg/kg (t=6.88, **P<0.001**)
Morphine + 5.6 mg/kg Mitragynine	$F_{1,64}$=0.00584 ; P=0.942	$F_{11,64}$=15.2; **P<0.001**	$F_{11,64}$=0.204; P=0.997	*Sex*: N/A. *Dose*: 0.32 mg/kg (t=4.17, **P=0.001**), 0.56 mg/kg (t=5.38, **P<0.001**), 1.0 mg/kg (t=6.29, **P<0.001**), 1.78 mg/kg (t=7.16, **P<0.001**), 3.2 mg/kg (t=7.19, **P<0.001**), 5.6 mg/kg (t=7.15, **P<0.001**), 10 mg/kg (t=5.72, **P<0.001**)
Rate-Decreasing Effects				
Drug	Sex	Dose (mg/kg)	Interaction	*Post-Hoc* Test
Morphine[#]	$F_{1,54}$=3.37; P=0.116	$F_{9,54}$=41.3; **P<0.001**	$F_{9,54}$=0.591; P=0.798	*Sex*: 10.0 mg/kg (t=2.51, **P=0.015**). *Dose*: 10.0 mg/kg (t=7.17, **P<0.001**), 17.8 mg/kg (t=9.41, **P<0.001**), 32 mg/kg (t=10.1, **P<0.001**), 56 mg/kg (t=10.1, **P<0.001**)
Morphine[##]	$F_{1,54}$=0.0236; P=0.883	$F_{9,54}$=18.9; **P<0.001**	$F_{9,54}$=0.543; P=0.837	*Sex*: N/A. *Dose*: 17.8 mg/kg (t=5.00, **P<0.001**), 32 mg/kg (t=6.57, **P<0.001**), 56 mg/kg (t=7.37, **P<0.001**)
Mitragynine Substitution for Morphine	$F_{1,30}$= 0.343; P=0.580	$F_{5,30}$= 6.76; **P<0.001**	$F_{5,30}$= 0.149; P=0.979	*Sex*: N/A. *Dose*: 56 mg/kg (t=4.85, **P<0.001**)
Fentanyl Substitution for Morphine	$F_{1,54}$= 0.0184; P=0.896	$F_{9,54}$= 26.9; **P<0.001**	$F_{9,54}$= 0.425; P=0.916	*Sex*: N/A. *Dose*: 0.056 mg/kg (t=3.66, **P=0.005**), 0.178 mg/kg (t=7.55, **P<0.001**), 0.32 mg/kg (t=10.2, **P<0.001**)
Buprenorphine Substitution for Morphine	$F_{1,42}$= 2.66; P=0.154	$F_{7,42}$= 4.88; **P<0.001**	$F_{7,42}$= 1.52; P=0.187	*Sex*: 0.56 mg/kg (t=2.39, **P=0.021**). *Dose*: 0.32 mg/kg (t=3.70, **P=0.004**), 0.56 mg/kg (t=4.21, **P<0.001**)

Nalbuphine Substitution for Morphine	$F_{1,54}$= 0.0491; P=0.832	$F_{9,54}$= 14.9; **P<0.001**	$F_{9,54}$= 0.465; P=0.892	*Sex*: N/A. *Dose*: 100 mg/kg (t=5.39, **P<0.001**), 178 mg/kg (t=7.67, **P<0.001**)
7-Hydroxymitragynine Substitution for Morphine	$F_{1,59}$= 0.909; P=0.377	$F_{10,59}$= 19.6; **P<0.001**	$F_{10,59}$= 0.731; P=0.693	*Sex*: N/A. *Dose*: 1.78 mg/kg (t=3.37, **P=0.013**), 3.2 mg/kg (t=5.24, **P<0.001**), 5.6 mg/kg (t=5.95, **P<0.001**), 10 mg/kg (t=7.10, **P<0.001**), 17.8 mg/kg (t=7.12, **P<0.001**)
U69,593 Substitution for Morphine	$F_{1,36}$=1.80; P=0.228	$F_{6,36}$=24.3; **P<0.001**	$F_{6,36}$=1.28; P=0.289	*Sex*: 0.56 mg/kg (t=2.23, **P=0.031**). *Dose*: 1.0 mg/kg (t=3.17, **P=0.019**), 1.78 mg/kg (t=4.73, **P<0.001**), 3.2 mg/kg (t=6.34, **P<0.001**), 5.6 mg/kg (t=8.40, **P<0.001**)
SNC 80 Substitution for Morphine	$F_{1,18}$=0.431; P=0.536	$F_{3,18}$=4.80; **P=0.013**	$F_{3,18}$=0.280; P=0.839	*Sex*: N/A. *Dose*: 100 mg/kg (t=3.48, **P=0.008**)
Morphine + 0.32 mg/kg Naltrexone	$F_{1,36}$=7.12; **P=0.037**	$F_{6,36}$=9.82; **P<0.001**	$F_{6,36}$=0.692; P=0.657	*Sex*: 32 mg/kg (t=2.92, **P=0.006**), *Dose*: 32 mg/kg (t=3.11, **P=0.022**), 56 mg/kg (t=4.49, **P<0.001**)
7-Hydroxymitragynine Substitution for Morphine + 0.032 mg/kg naltrexone	$F_{1,48}$= 0.199; P=0.671	$F_{8,48}$= 19.8; **P<0.001**	$F_{8,48}$= 0.810; P=0.597	*Sex*: N/A. *Dose*: 3.2 mg/kg (t=3.70, **P=0.004**), 5.6 mg/kg (t=5.37, **P<0.001**), 10 mg/kg (t=6.61, **P<0.001**), 17.8 mg/kg (t=7.76, **P<0.001**)
Morphine + 1.78 mg/kg Nalbuphine	$F_{1,72}$= 0.233; P=0.647	$F_{12,72}$= 27.1; **P<0.001**	$F_{12,72}$= 1.13; P=0.347	*Sex*: N/A. *Dose*: 17.8 mg/kg (t=4.24, **P<0.001**), 32 mg/kg (t=10.0, **P<0.001**), 56 mg/kg (t=10.7, **P<0.001**)
Morphine + 5.6 mg/kg Mitragynine	$F_{1,84}$= 0.0873; P=0.778	$F_{14,84}$= 21.0; **P<0.001**	$F_{14,84}$= 0.878; P=0.585	*Sex*: 0.32 mg/kg (t=2.69, **P=0.009**). *Dose*: 10 mg/kg (t=3.58, **P=0.008**), 17.8 mg/kg (t=6.51, **P<0.001**), 32 mg/kg (t=7.26, **P<0.001**), 56 mg/kg (t=7.32, **P<0.001**)

Maximum Possible Effects				
Drug	Sex	Dose (mg/kg)	Interaction	*Post-Hoc* Test
Morphine[#]	$F_{1,54}$= 0.518; P=0.499	$F_{9,54}$= 93.4; **P<0.001**	$F_{9,54}$= 1.77; P= 0.097	*Sex*: 32 mg/kg (t=3.83, **P<0.001**). *Dose*: 32 mg/kg (t=10.1, **P<0.001**), 56 mg/kg (t=20.0, **P<0.001**)
Morphine[##]	$F_{1,54}$= 0.637; P= 0.455	$F_{9,54}$= 86.3; **P<0.001**	$F_{9,54}$= 0.525; P=0.850	*Sex*: N/A. *Dose*: 32 mg/kg (t=8.96, **P<0.001**), 56 mg/kg (t=20.4, **P<0.001**)
Mitragynine Substitution for Morphine	$F_{1,30}$= 0.675; P=0.443	$F_{5,30}$= 0.566; P=0.725	$F_{5,30}$= 1.32; P= 0.283	*Sex*: N/A. *Dose*: N/A
Fentanyl Substitution for Morphine	$F_{1,54}$= 4.45; P=0.079	$F_{9,54}$= 62.7; **P<0.001**	$F_{9,54}$= 1.54; P=0.158	*Sex*: 0.056 mg/kg (t=2.19, **P=0.034**), 0.1 mg/kg (t=3.31, **P=0.002**), 0.178 mg/kg (t=2.15, **P=0.037**). *Dose*: 0.1 mg/kg (t=8.38, **P<0.001**), 0.178 mg/kg (t=12.5, **P<0.001**), 0.32 mg/kg (t=14.5, **P<0.001**)
Buprenorphine Substitution for Morphine	$F_{1,42}$= 0.0129; P=0.913	$F_{7,42}$= 2.26; **P=0.048**	$F_{7,42}$= 0.653; P=0.710	*Sex*: N/A. *Dose*: 0.32 mg/kg (t=3.05, **P=0.028**), 0.056 mg/kg (t=3.59, **P=0.006**), 0.178 mg/kg (t=2.85, **P=0.047**)
Nalbuphine Substitution for Morphine	$F_{1,54}$= 0.192; P=0.676	$F_{9,54}$= 1.04; P=0.418	$F_{9,54}$= 0.768; P=0.646	N/A
7-Hydroxymitragynine Substitution for Morphine	$F_{1,60}$= 0.132; P=0.729	$F_{10,60}$= 30.8; **P<0.001**	$F_{10,60}$= 1.38; P=0.213	*Sex*: 10 mg/kg (t=3.00, **P=0.004**). *Dose*: 10 mg/kg (t=8.83, **P<0.001**), 17.8 mg/kg (t=9.99, **P<0.001**)
U69,593 Substitution for Morphine	$F_{1,36}$=2.75; P=0.148	$F_{6,36}$=15.1; **P<0.001**	$F_{6,36}$=2.38; **P=0.048**	*Sex*: 5.6 mg/kg (t=3.62, **P=0.001**). *Dose*: 3.2 mg/kg (t=5.50, **P<0.001**), 5.6 mg/kg (t=6.87, **P<0.001**)

SNC 80 Substitution for Morphine	$F_{1,18}$=0.595; P=0.470	$F_{3,18}$=0.886; P=0.467	$F_{3,18}$=1.13; P=0.365	N/A
Morphine + 0.032 mg/kg naltrexone	$F_{1,36}$=0.938; P=0.370	$F_{6,36}$=3.00; **P=0.018**	$F_{6,36}$=3.53; **P=0.008**	*Sex*: 56.0 mg/kg (t=3.340, **P=0.003**). *Dose*: N/A
7-Hydroxymitragynine Substitution for Morphine + 0.032 mg/kg naltrexone	$F_{1,48}$= 4.80; P=0.071	$F_{8,48}$= 0.809; P=0.598	$F_{8,48}$= 0.916; P=0.512	N/A
Morphine + 1.78 mg/kg Nalbuphine	$F_{1,72}$= 0.797; P=0.406	$F_{12,72}$= 4.61; **P<0.001**	$F_{12,72}$= 1.67; P=0.093	*Sex*: 10.0 mg/kg (t=3.30, **P=0.002**), 32.0 mg/kg (t=2.23, **P=0.03**). *Dose*: 56 mg/kg (t=5.30, **P<0.001**)
Morphine + 5.6 mg/kg Mitragynine	$F_{1,84}$= 3.54; P=0.109	$F_{14,84}$= 9.59; **P<0.001**	$F_{14,84}$=1.00; P=0.461	*Sex*: 0.1 mg/kg (t=2.21, **P=0.03**). *Dose*: 56 mg/kg (t=6.69, **P<0.001**)
Rats Trained with Mitragynine				
Discriminative-Stimulus Effects				
Drug	Sex	Dose (mg/kg)	Interaction	*Post-Hoc* Test
Mitragynine[#]	$F_{1,30}$=1.29; P=0.300	$F_{5,30}$=31.6; **P<0.001**	$F_{5,30}$=0.899; P=0.495	*Sex*: N/A. *Dose*: 10 mg/kg (t=4.32, **P<0.001**), 17.8 mg/kg (t=6.98, **P<0.001**), 32 mg/kg (t=9.81, **P<0.001**)
Mitragynine[##]	$F_{1,31}$=0.142; P=0.718	$F_{6,31}$=31.1; **P<0.001**	$F_{6,31}$=3.13; **P=0.016**	*Sex*: 10.0 mg/kg (t=3.19, **P=0.004**). *Dose*: 10 mg/kg (t=5.04, **P<0.001**), 17.8 mg/kg (t=8.56, **P<0.001**), 32 mg/kg (t=9.98, **P<0.001**), 56 mg/kg (t=7.10, **P<0.001**)

Morphine Substitution for Mitragynine	$F_{1,40}$=0.160; P=0.703	$F_{7,40}$= 6.62; **P<0.001**	$F_{7,40}$= 1.01; P=0.441	*Sex*: N/A. *Dose*: 10 mg/kg (t=4.46, **P<0.001**), 17.8 mg/kg (t=4.63, **P<0.001**)
Fentanyl Substitution for Mitragynine	$F_{1,37}$=8.54; **P=0.026**	$F_{7,37}$=10.7; **P<0.001**	$F_{7,37}$=2.41; **P=0.039**	*Sex*: 0.032 mg/kg (t=2.55, **P=0.016**), 0.056 mg/kg (t=2.46, **P=0.020**), 0.1 mg/kg (t=3.31, **P=0.002**), 0.178 mg/kg (t=2.20, **P=0.036**). *Dose*: 0.0178 mg/kg (t=3.59, **P=0.007**), 0.032 mg/kg (t=5.55, **P<0.001**), 0.056 mg/kg (t=5.62, **P<0.001**), 0.1 mg/kg (t=3.73, **P=0.004**), 0.178 mg/kg (t=4.20, **P=0.001**)
Buprenorphine Substitution for Mitragynine	$F_{1,38}$ = 2.30; P=0.179	$F_{7,38}$= 11.3; **P<0.001**	$F_{7,38}$= 1.43; P=0.224	*Sex*: 0.1 mg/kg (t=2.94, **P=0.006**). *Dose*: 0.1 mg/kg (t=3.95, **P=0.002**), 0.178 mg/kg (t=4.62, **P<0.001**), 0.32 mg/kg (t=5.23, **P<0.001**), 0.56 mg/kg (t=4.69, **P<0.001**)
Nalbuphine Substitution for Mitragynine	$F_{1,48}$ = 0.110; P=0.751	$F_{8,48}$= 17.5; **P<0.001**	$F_{8,48}$= 0.619; P=0.758	*Sex*: N/A. *Dose*: 5.6 mg/kg (t=4.33, **P<0.001**), 10 mg/kg (t=5.66, **P<0.001**), 17.8 mg/kg (t=6.82, **P<0.001**), 32 mg/kg (t=7.10, **P<0.001**), 56 mg/kg (t=7.11, **P<0.001**)
7-Hydroxymitragynine Substitution for Mitragynine	$F_{1,34}$ = 1.91; P=0.216	$F_{6,34}$= 34.6; **P<0.001**	$F_{6,34}$= 0.846; P=0.544	*Sex*: N/A. *Dose*: 0.32 mg/kg (t=7.96, **P<0.001**), 0.56 mg/kg (t=8.01, **P<0.001**), 1.0 mg/kg (t=7.43, **P<0.001**), 1.78 mg/kg (t=8.97, **P<0.001**)
U69,593 Substitution for Mitragynine	$F_{1,29}$=4.84; P=0.070	$F_{5,29}$=1.50; P=0.221	$F_{5,29}$=1.22; P=0.327	N/A
SNC 80 Substitution for mitragynine	$F_{1,12}$ = 2.02; P=0.205	$F_{2,12}$= 4.33; **P=0.038**	$F_{2,12}$= 2.00; P=0.178	*Sex*: 100 mg/kg (t=2.43, **P=0.026**). *Dose*: 100 mg/kg (t=2.59, **P=0.047**)

Mitragynine + 0.032 mg/kg naltrexone	$F_{1,17}$= 0.292; P=0.608	$F_{3,17}$= 1.82; P=0.181	$F_{3,17}$= 0.463; P=0.712	N/A
Rate-Decreasing Effects				
Drug	Sex	Dose (mg/kg)	Interaction	*Post-Hoc* Test
Mitragynine[#]	$F_{1,36}$=2.03; P=0.205	$F_{6,36}$=8.65; **P<0.001**	$F_{6,36}$=1.25; P=0.304	*Sex*: 56 mg/kg (t=2.71, **P=0.010**). *Dose*: 56 mg/kg (t=4.99, **P<0.001**)
Mitragynine[##]	$F_{1,36}$=0.301; P=0.603	$F_{6,36}$=6.02; **P<0.001**	$F_{6,36}$=0.430; P=0.854	*Sex*: N/A. *Dose*: 56 mg/kg (t=4.76, **P<0.001**)
Morphine Substitution for Mitragynine	$F_{1,54}$=0.224; P=0.653	$F_{9,54}$=23.8; **P<0.001**	$F_{9,54}$=3.45; **P=0.002**	*Sex*: 0.56 mg/kg (t=2.02, **P=0.048**), 3.2 mg/kg (t=2.07, **P=0.043**), 10.0 mg/kg (t=2.50, **P=0.015**), 17.8 mg/kg (t=2.72, **P=0.008**). *Dose*: 17.8 mg/kg (t=4.73, **P<0.001**), 32 mg/kg (t=7.40, **P<0.001**), 56 mg/kg (t=8.30, **P<0.001**)
Fentanyl Substitution for Mitragynine	$F_{1,48}$=0.280; P=0.616	$F_{8,48}$=10.4; **P<0.001**	$F_{8,48}$=0.985; P=0.459	*Sex*: N/A. *Dose*: 0.178 mg/kg (t=4.78, **P<0.001**), 0.32 mg/kg (t=7.32, **P<0.001**)
Buprenorphine Substitution for Mitragynine	$F_{1,60}$=0.662; P=0.447	$F_{10,60}$=13.1; **P<0.001**	$F_{10,60}$=0.716; P=0.706	*Sex*: N/A. *Dose*: 0.56 mg/kg (t=5.50, **P<0.001**)
Nalbuphine Substitution for Mitragynine	$F_{1,42}$=2.44; P=0.169	$F_{7,42}$=11.0; **P<0.001**	$F_{7,42}$=0.953; P=0.478	*Sex*: N/A. *Dose*: 178 mg/kg (t=6.30, **P<0.001**

7-Hydroxymitragynine Substitution for Mitragynine	$F_{1,60}$=0.166; P=0.698	$F_{10,60}$=14.5; **P<0.001**	$F_{10,60}$=0.274; P=0.985	*Sex*: N/A. *Dose*: 3.2 mg/kg (t=4.38, **P<0.001**), 5.6 mg/kg (t=4.30, **P<0.001**), 10 mg/kg (t=5.72, **P<0.001**), 17.8 mg/kg (t=6.36, **P<0.001**)
U69,593 Substitution for Mitragynine	$F_{1,36}$=1.20; P=0.315	$F_{6,36}$=23.2; **P<0.001**	$F_{6,36}$=1.65; P=0.162	*Sex*: 1.78 mg/kg (t=2.57, **P=0.014**). *Dose*: 1.78 mg/kg (t=3.01, **P=0.023**), 3.2 mg/kg (t=6.58, **P<0.001**), 5.6 mg/kg (t=8.32, **P<0.001**)
SNC 80 Substitution for mitragynine	$F_{1,12}$=0.0732; P=0.796	$F_{2,12}$=11.6; **P=0.002**	$F_{2,12}$=3.53; P=0.062	*Sex*: 100 mg/kg (t=2.27, **P=0.035**). *Dose*: 100 mg/kg (t=3.27, **P=0.013**)
Mitragynine + 0.032 mg/kg naltrexone	$F_{1,18}$=1.14; P=0.327	$F_{3,18}$=3.51; **P=0.037**	$F_{3,18}$=0.299; P=0.826	*Sex*: N/A. *Dose*: 56 mg/kg (t=2.87, **P=0.03**)
Maximum Possible Effects				
Drug	Sex	Dose (mg/kg)	Interaction	*Post-Hoc* Test
Mitragynine[#]	$F_{1,36}$=1.04; P=0.348	$F_{6,36}$=2.08; P=0.080	$F_{6,36}$=0.531; P=0.781	N/A
Mitragynine[##]	$F_{1,36}$= 0.703; P=0.434	$F_{6,36}$= 3.71; **P=0.006**	$F_{6,36}$=0.907; P=0.501	N/A
Morphine Substitution for Mitragynine	$F_{1,54}$=0.990; P=0.358	$F_{9,54}$=27.1; **P<0.001**	$F_{9,54}$=0.109; P=0.999	*Sex*: N/A. *Drug*: 32 mg/kg (t=6.36, **P<0.001**), 56 mg/kg (t=10.8, **P<0.001**)
Fentanyl Substitution for Mitragynine	$F_{1,48}$= 0.212; P=0.661	$F_{8,48}$= 82.9; **P<0.001**	$F_{8,48}$= 0.403; P=0.913	*Sex*: N/A. *Dose*: 0.056 mg/kg (t=4.28, **P<0.001**), 0.1 mg/kg (t=12.4, **P<0.001**), 0.178 mg/kg (t=14.0, **P<0.001**), 0.32 mg/kg (t=15.3, **P<0.001**)

Buprenorphine Substitution for Mitragynine	$F_{1,60}$=0.132; P=0.728	$F_{10,60}$=2.34; **P=0.021**	$F_{10,60}$=0.798; P=0.631	N/A
Nalbuphine Substitution for Mitragynine	$F_{1,42}$=0.177; P=0.689	$F_{7,42}$=1.38; P=0.239	$F_{7,42}$=0.589; P=0.761	*Sex*: N/A. *Dose*: 1.0 mg/kg (t=3.22, **P=0.02**), 5.6 mg/kg (t=3.66, **P=0.005**)
7-Hydroxymitragynine Substitution for Mitragynine	$F_{1,60}$=1.19; P=0.318	$F_{10,60}$=13.1; **P<0.001**	$F_{10,60}$=1.33; P=0.238	*Sex*: 10 mg/kg (t=2.98, **P=0.004**). *Dose*: 1.0 mg/kg (t=7.23, **P<0.001**)
U69,593 Substitution for Mitragynine	$F_{1,36}$=0.177; P=0.688	$F_{6,36}$=15.5; **P<0.001**	$F_{6,36}$=0.465; P=0.829	Sex: N/A. *Dose*: 5.6 mg/kg (t=6.63, **P<0.001**)
SNC 80 Substitution for mitragynine	$F_{1,12}$=0.753; P=0.419	$F_{2,12}$=3.53; P=0.062	$F_{2,12}$=01.29; P=0.311	N/A
Mitragynine + 0.032 mg/kg naltrexone	$F_{1,18}$=3.70; P=0.103	$F_{3,18}$=0.582; P=0.635	$F_{3,18}$=0.834; P=0.492	N/A

Supplemental Table 3 ED_{50} values (95% CIs) for the discriminative-stimulus, rate-decreasing, and antinociceptive effects of various compounds in separate groups of males and females rats trained to discriminate either 3.2 mg/kg morphine or 32 mg/kg mitragynine as shown in Supplemental Figures 6 and 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, #First assessment, ##Reassessment.

Test drug	ED_{50} (95% CIs)			Potency Ratio	
	Discrimination	Response Rate	Maximum Possible Effect	Rate-Decreasing / Discrimination	Antinociceptive / Discrimination
Rats Trained to Discriminate an Injection of 3.2 mg/kg Morphine from its Vehicle					
Fentanyl (Females)	0.0300 (0.0168 — 0.0448)	0.175 (0.146 — 0.215)	0.108 (0.0626 — 0.145)	5.83 (3.26 — 12.8)	3.6 (1.40 — 8.63)
Fentanyl (Males)	0.0128 (0.00427 — 0.0202)	0.170 (0.135 — 0.222)	0.168 (0.132 — 0.211)	13.3 (6.68 — 52.0)	13.1 (6.53 — 49.4)
Mitragynine (Females)	25.3 (12.6 — 47.9)	44.6 (28.9 — 153)	ND* [≤12.7% (12.2%) @ 5.6 mg/kg]	1.76 (0.603 — 12.1)	Not Applicable
Mitragynine (Males)	33.9 (19.9 — 104)	46.5 (36.8 — 66.8)	ND* [≤5.49% (3.58%) @ 32 mg/kg]	1.37 (0.354 — 3.36)	Not Applicable

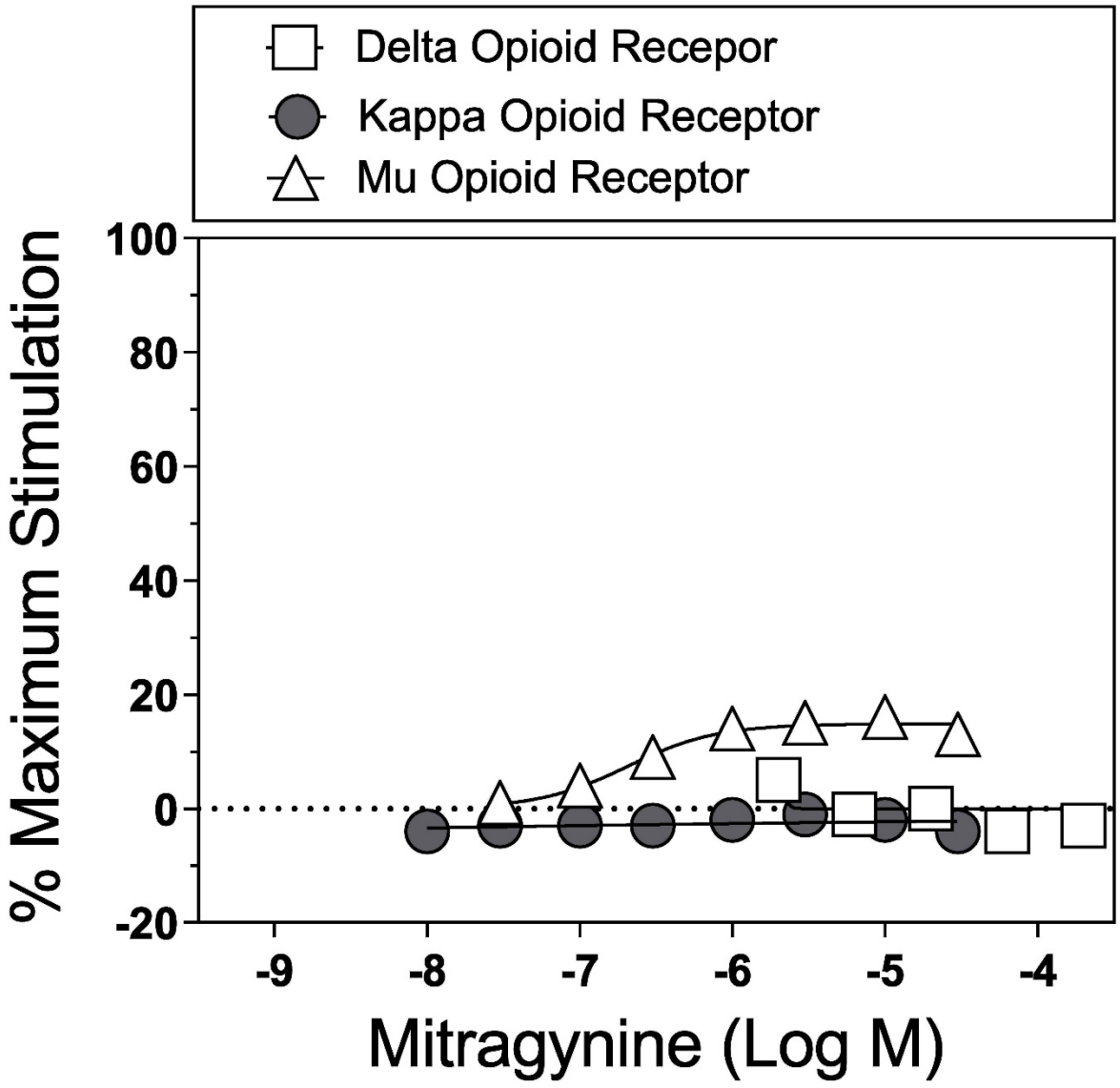
Morphine (Females)	#1.55 (0.876 — 2.14), ##1.74 (1.42 — 2.08)	#15.2 (10.7 — 20.3), ##21.6 (14.4 — 30.1)	#38.2 (35.9 — 40.6), ##35.1 (30.7 — 39.6)	#9.81 (5.00 — 23.2), ##12.4 (6.92 — 21.2)	#24.6 (16.8 — 46.3), ##20.2 (14.8 — 27.9)
Morphine (Males)	#2.11 (1.77 — 2.58), ##1.42 (0.589 — 2.05)	#12.0 (6.73 — 16.6), ##21.0 (15.0 — 27.8)	#35.4 (29.2 — 41.6), ##37.1 (34.6 — 39.7)	#5.69 (2.61 — 9.38), ##14.8 (7.32 — 47.2)	#16.8 (11.3 — 23.5), ##26.1 (16.8 — 67.4)
Rats Trained to Discriminate an Injection of 32 mg/kg Mitragynine from its Vehicle					
Fentanyl (Females)	0.0172 (0.0119 — 0.0229)	0.164 (0.126 — 0.223)	0.121 (0.0794 — 0.163)	9.54 (5.5 — 18.7)	7.03 (3.47 — 13.7)
Fentanyl (Males)	0.0743 (0.0479 — 0.154)	0.177 (0.131 — 0.260)	0.115 (0.0792 — 0.151)	2.38 (0.85 — 5.43)	1.55 (0.51 — 3.15)
Mitragynine (Females)	#13.4 (9.03 — 17.6), ##11.9 (6.97 — 16.2)	#36.2 (29.7 — 46.1), ##45.7 (35.4 — 70.5)	ND* [up to #12.4% (8.82%) and ##9.80% (7.95%) @ 32 and 56 mg/kg]	#2.70 (1.69 — 5.11), ##3.84 (2.19 — 10.1)	Not Applicable

Mitragynine (Males)	[#]16.8 (13.8 — 20.3), [##]13.6 (7.37 — 19.9)	[#]64.5 (43.4 — 575), [##]49.0 (33.6 — 167)	ND* [up to [#]10.1% (6.35%) and [##]6.21% (6.48%) @ 56 mg/kg]	[#]3.84 (2.14 — 41.7), [##]3.60 (1.69 — 22.7)	Not Applicable

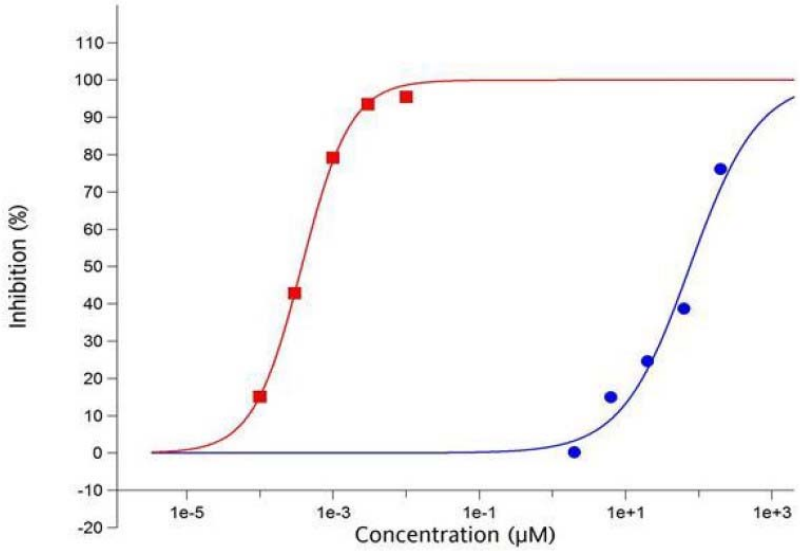
Supplemental Table 4 Comparison of potency ratios of various compounds tested to produce the discriminative stimulus, rate-decreasing, and antinociceptive effects in mitragynine-trained rats relative to those in morphine-trained rats. Each value (95%CIs in parentheses) is for a combination of females with males unless described and calculated as a division of the ED_{50} values in mitragynine-trained rats by the ED_{50} values in morphine-trained rats as shown in Table 4. The sample sizes are described in each figure legend (Figures 3—6). Significant differences are bold.

Test compound	Discriminative Stimulus	Response Rate	Antinociception
7-Hydroxymitragynine	1.51 (0.202 — 8.54)	1.17 (0.617 — 2.26)	1.55 (1.13 — 2.24)
Buprenorphine	2.20 (0.551 — 6.82)	1.17 (0.604 — 2.14)	Not Applicable
Fentanyl	2.30 (0.390 — 8.16)	0.988 (0.693 — 1.43)	0.849 (0.559 — 1.28)
Fentanyl (Females)	0.573 (0.266 — 1.36)	0.937 (0.586 — 1.53)	1.12 (0.548 — 2.60)
Fentanyl (Males)	**5.81 (2.37 — 36.1)**	1.04 (0.590 — 1.93)	0.685 (0.375 — 1.14)
Mitragynine	First: 0.510 (0.227 — 0.936) Reassessment: 0.432 (0.167 — 0.851)	First: 1.01 (0.594 — 1.66) Reassessment: 1.03 (0.596 — 1.80)	Not Applicable
Mitragynine (Females)	First: 0.530 (0.189 — 1.40) Reassessment: 0.470 (0.146 — 1.29)	First: 0.812 (0.194 — 1.60) Reassessment: 1.02 (0.231 — 2.44)	Not Applicable
Mitragynine (Males)	First: 0.496 (0.133 — 1.02) Reassessment: 0.401 (0.0709 — 1.00)	First: 1.39 (0.650 — 15.6) Reassessment: 1.05 (0.503 — 4.54)	Not Applicable

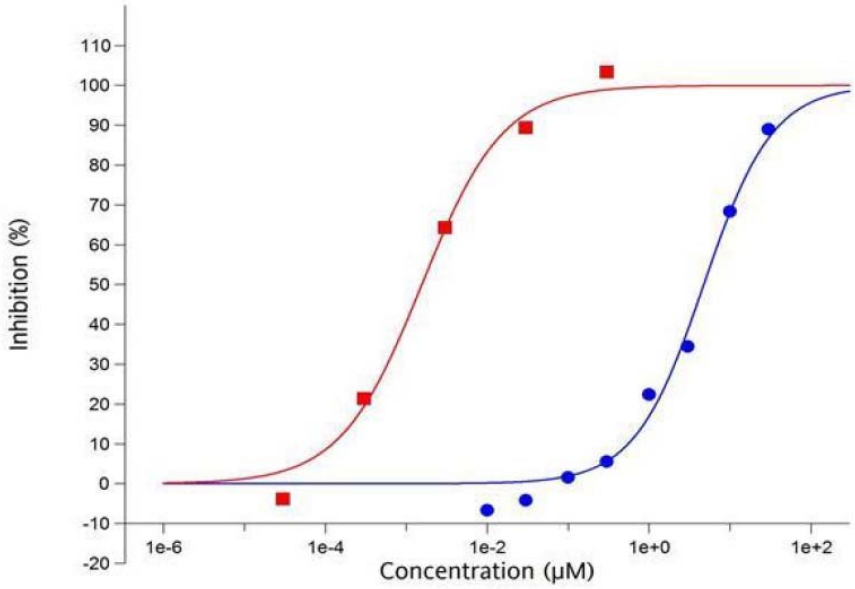
Morphine	First: **8.49 (4.73 — 23.6)** Reassessment: **9.81 (5.45 — 27.9)**	First: 1.81 (1.15 — 2.88) Reassessment: 1.16 (0.737 — 1.82)	First: 1.01 (0.822 — 1.25) Reassessment: 1.07 (0.860 — 1.32)
Morphine (females)	First: **4.97 (2.48 — 13.0)** Reassessment: **4.43 (2.55 — 8.03)**	First: 1.33 (0.428 — 2.73) Reassessment: 0.935 (0.288 — 2.03)	First: 0.955 (0.741 — 1.26) Reassessment: 1.04 (0.760 — 1.48)
Morphine (males)	First: **8.82 (4.38 — 28.9)** Reassessment: **13.1 (5.51 — 86.9)**	First: 2.43 (1.33 — 5.57) Reassessment: 1.39 (0.795 — 2.50)	First: 0.983 (0.673 — 1.49) Reassessment: 0.938 (0.705 — 1.26)
Nalbuphine	0.912 (0.391 — 3.87)	1.35 (0.955 — 1.99)	Not Applicable
SNC80	Not Applicable	Not Applicable	Not Applicable
U69,593	Not Applicable	1.08 (0.747 — 1.59)	1.13 (0.750 — 1.66)



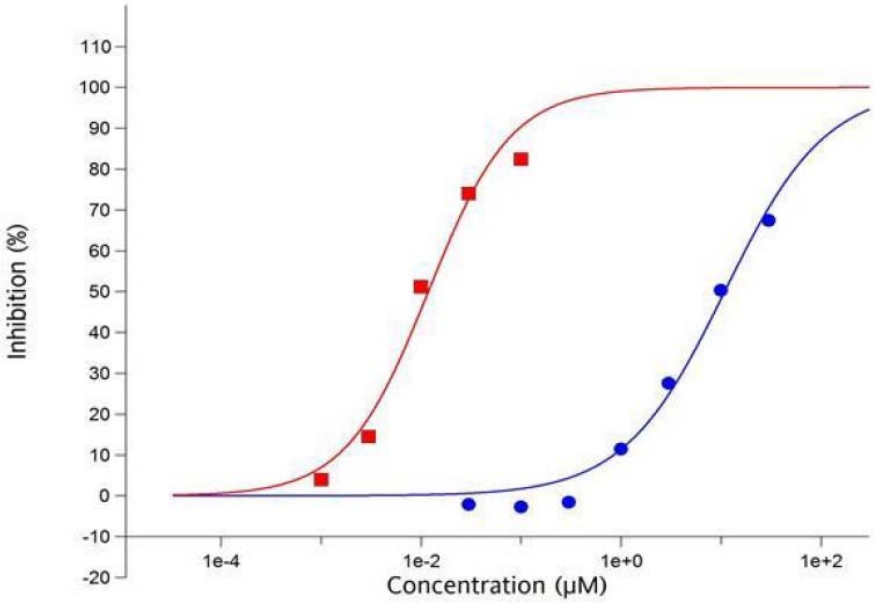
Supplemental Figure 1. Concentration-effect curves of mitragynine in stimulating [^{35}S]GTPγS binding in CHO cell lines stably expressing the human delta-, kappa-, and mu-opioid receptors from Eurofins Cerep (Celle l'Evescault, France). The EC_{50} values of the reference agonists DPDPE, U69,593, and DAMGO at the delta-, kappa-, and mu-opioid receptors were 40.0, 11.1, and 7.15 nM, respectively.



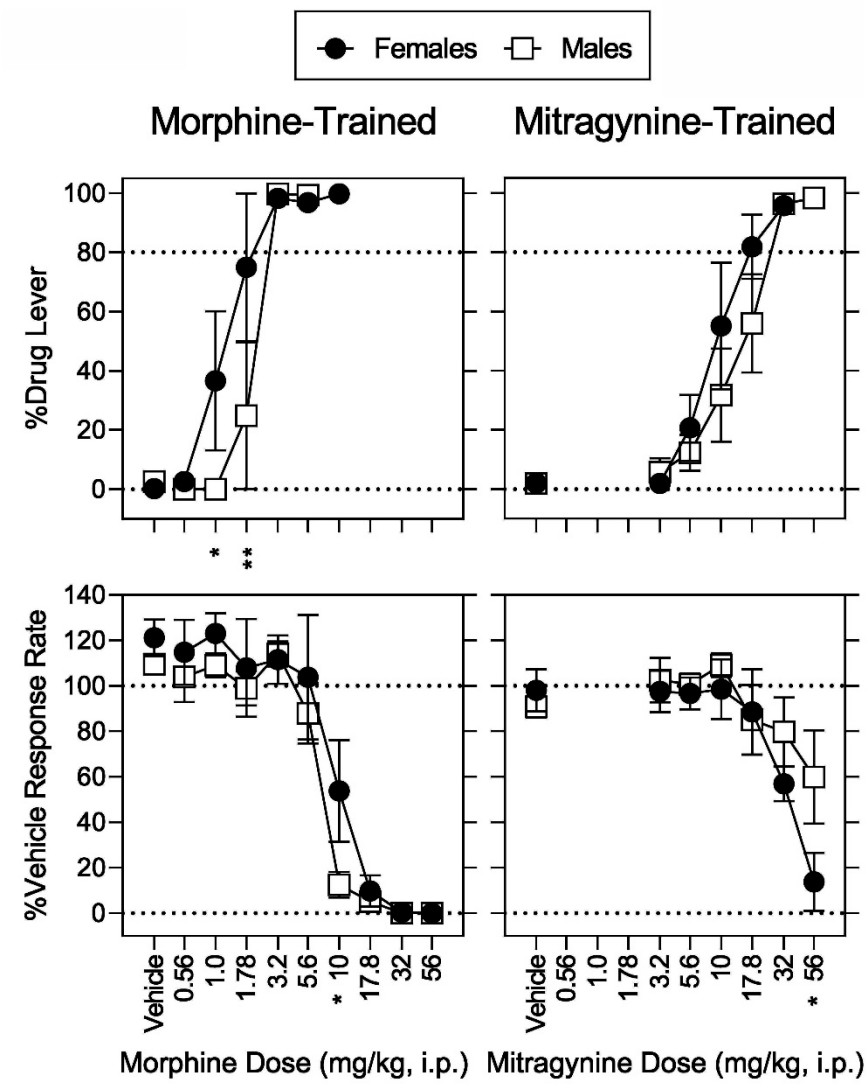
Supplemental Figure 2. Antagonism of DPDPE agonist effects (unspecified from Eurofins Cerep) by mitragynine (blue circles) and naltrindole (red squares) at the delta-opioid receptor using the [^{35}S]GTPγS binding from Eurofins Cerep (Celle l'Evescault, France). The IC_{50} values of mitragynine and naltrindole were 75.7 µM and 0.37 nM, respectively.



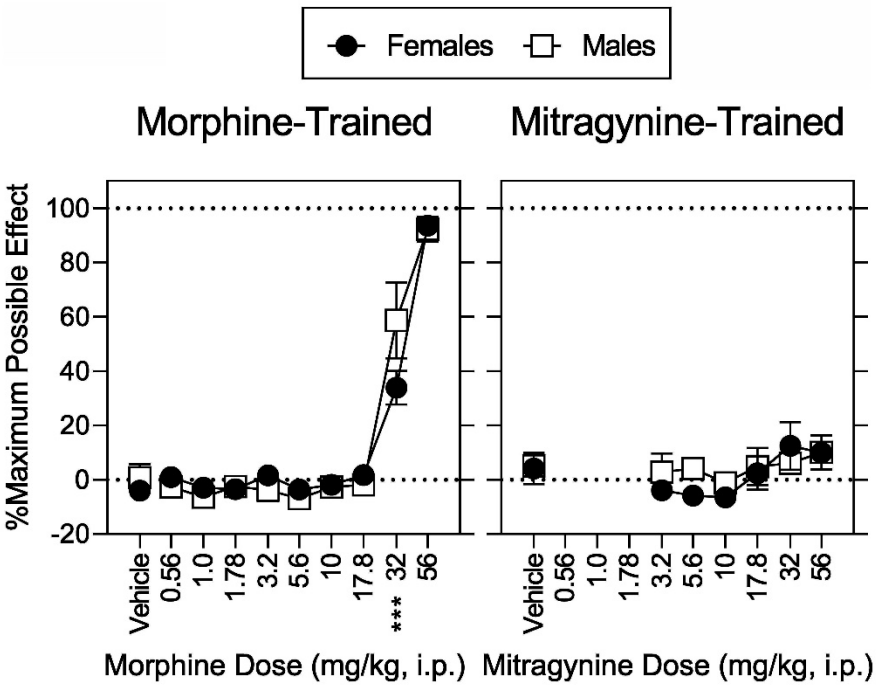
Supplemental Figure 3. Antagonism of U69,593 agonist effects (unspecified from Eurofins Cerep) by mitragynine (blue circles) and nor-binaltorphimine (red squares) at the kappa-opioid receptor using the [^{35}S]GTPγS binding from Eurofins Cerep (Celle l'Evescault, France). The IC_{50} of mitragynine and nor-binaltorphimine were 4.73 μM and 1.55 nM, respectively.



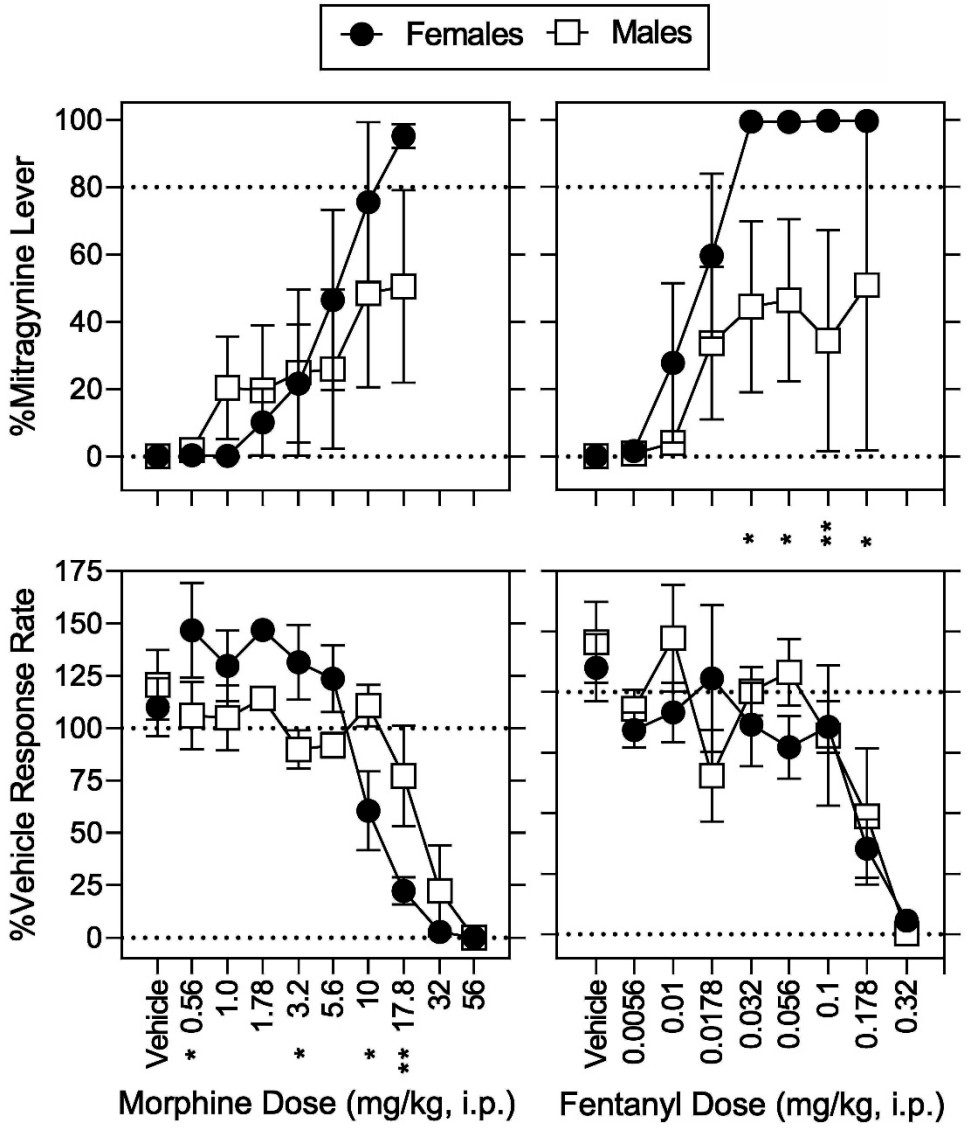
Supplemental Figure 4. Antagonism of DAMGO agonist effects (unspecified from Eurofins Cerep) by mitragynine (blue circles) and naltrexone (red squares) at the MOR using the [^{35}S]GTPγS binding from Eurofins Cerep (Celle l'Evescault, France). The IC_{50} values of mitragynine and naltrexone were 10.8 µM and 11.8 nM, respectively.



Supplemental Figure 5. Discriminative-stimulus effects in separate groups of rats discriminating either morphine or mitragynine, divided by sex. Abscissae: Vehicle and dose in mg/kg (i.p., log scale) for morphine (*left*) 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. Each point represents the mean ± SEM. Morphine and mitragynine were administered i.p., respectively, at 15 and 30 min before sessions. N=4 per data point except for %Drug Lever at 10 mg/kg morphine. *$p<0.05$ and **$p<0.01$ compared with females at each corresponding dose. Details for statistical analyses are shown in Tables 5 and 6 and Supplemental Tables 2 and 3.



Supplemental Figure 6. Antinociceptive effects in separate groups of rats discriminating either morphine or mitragynine, divided by sex. Ordinates: percentage of maximum possible antinociceptive effects. Abscissae: Vehicle and dose in mg/kg (i.p., log scale) for morphine (*left*) and mitragynine (*right*). Each point represents the mean ± SEM (N=4 per sex per data point). ***$p<0.001$ compared with females at each corresponding dose. Details for statistical analyses are shown in Tables 5 and 6 and Supplemental Tables 2 and 3.



Supplemental Figure 7. Effects of sex on substitution of morphine or fentanyl for mitragynine. Abscissae: Vehicle and drug dose in mg/kg (i.p., log scale). Ordinates: *Upper panels*, percentage of responses on mitragynine-appropriate lever; *lower panels*, percentage of mean rates of responding after vehicle administration during inter-test sessions. Each point represents the mean ± SEM (N=4 per sex per data point unless noted). Morphine and fentanyl were administered i.p. at 15 minutes before sessions while mitragynine at 30 minutes before sessions. *Upper left*: The mitragynine-like discriminative stimulus effects of morphine. Morphine doses; vehicle, and 0.56, 1.0, 1.78, 3.2, 5.6, 10, and 17.8 (two females and four males) mg/kg. *Lower left*: The rate-decreasing effects of morphine. Morphine doses; vehicle, and 0.56,

1.0, 1.78, 3.2, 5.6, 10, 17.8, 32, and 56 mg/kg. *Upper right*: The mitragynine-like discriminative-stimulus effects of fentanyl. Fentanyl doses; vehicle, and 0.0056, 0.01, 0.0178, 0.032, and 0.056, 0.1 (four females and three males), and 0.178 (three females and two males) mg/kg. *Lower right*: The rate-decreasing effects of fentanyl. Fentanyl doses; vehicle, and 0.0056, 0.01, 0.0178, 0.032, and 0.056, 0.1, and 0.178, and 0.32 mg/kg. *$p<0.05$, and **$p<0.01$ compared with females at each corresponding dose of morphine and fentanyl. Details for statistical analyses are shown in Tables 4 and Supplemental Tables 2 and 4.