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

Samuel Obeng[1,2,#], Jenny L. Wilkerson[1,#], Francisco Leon[2], Morgan E. Reeves[1], Luis F. Restrepo[1], Lea R. Gamez-Jimenez[1], Avi Patel[1], Anna E. Pennington[1], Victoria A. Taylor[1], Nicholas P. Ho[1], Tobias Braun[1], Morgan L. Crowley[2], Morgan R. Williamson[1], Victoria L.C. Pallares[1], Marco Mottinelli[2], Christopher R. McCurdy[2], Lance R. McMahon[1], and Takato Hiranita[1]*

Departments of Pharmacodynamics[1] and Medicinal Chemistry[2], College of Pharmacy, University of Florida

[#]These authors contributed equally

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

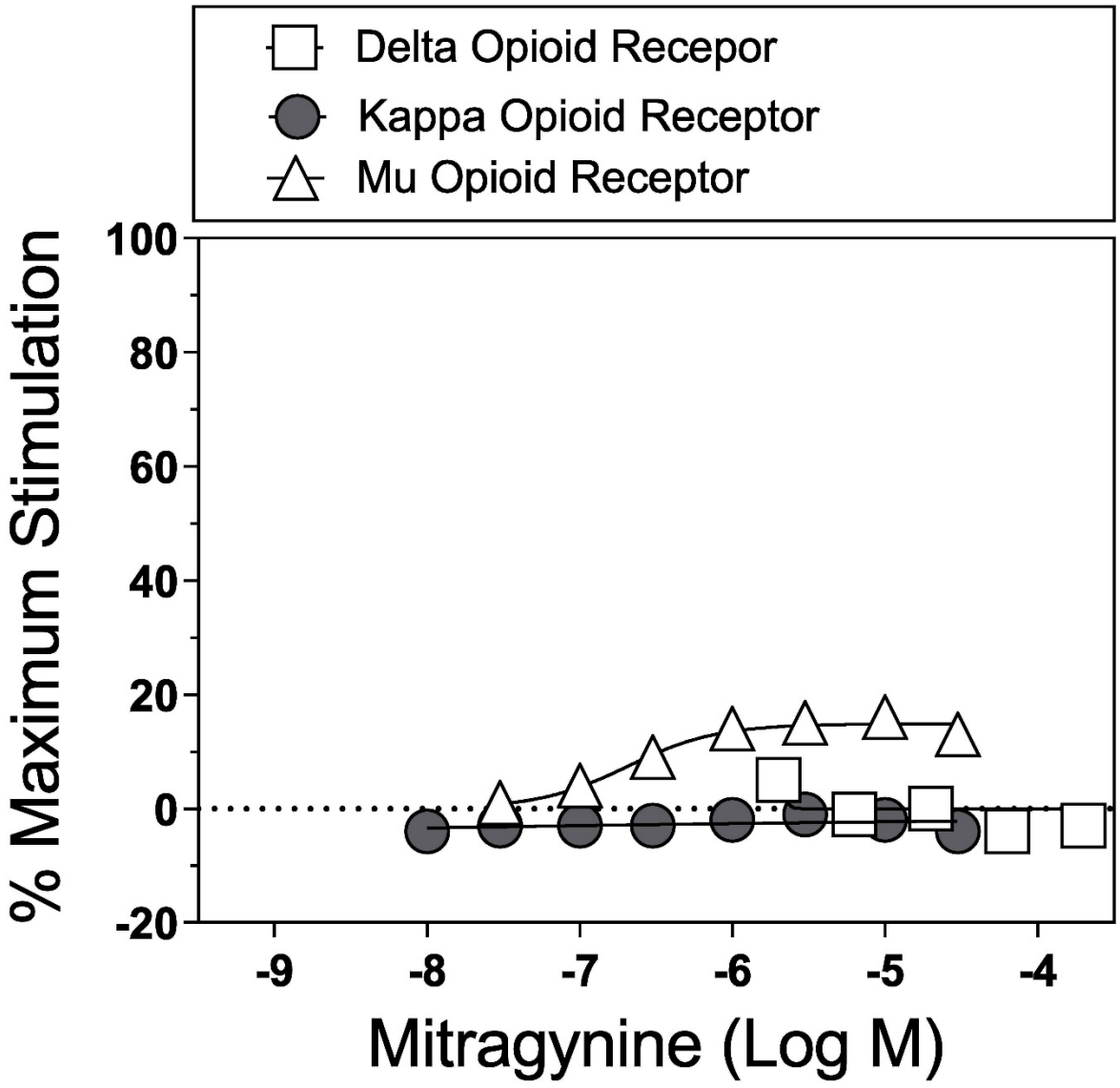
Morphine (Females)	#1.55 (0.876 — 2.14), ##1.74 (1.42 — 2.08)	#15.2 (10.7 — 20.3), ##21.6 (14.4 — 30.1)	#38.2 (35.9 — 40.6), ##35.1 (30.7 — 39.6)	#9.81 (5.00 — 23.2), ##12.4 (6.92 — 21.2)	#24.6 (16.8 — 46.3), ##20.2 (14.8 — 27.9)
Morphine (Males)	#2.11 (1.77 — 2.58), ##1.42 (0.589 — 2.05)	#12.0 (6.73 — 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

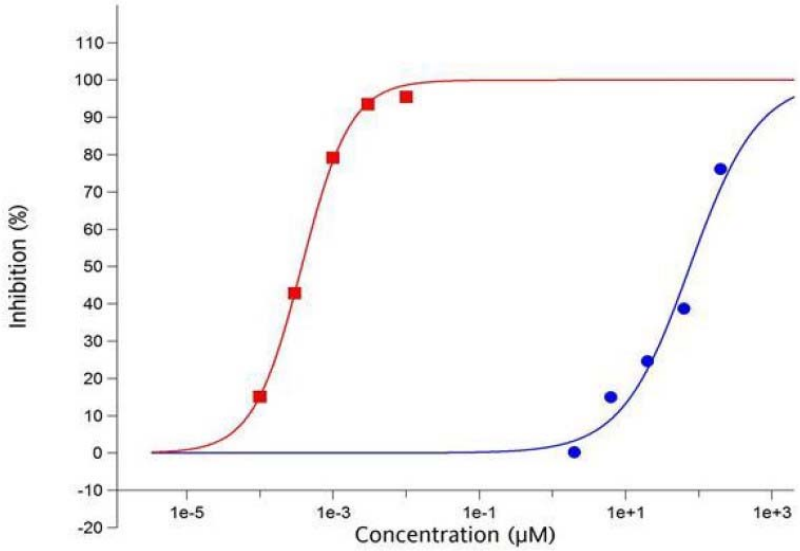
Supplemental Table 4 Comparison of potency ratios of various compounds tested to produce the discriminative stimulus, rate-decreasing, and antinociceptive effects in mitragynine-trained rats relative to those in morphine-trained rats. Each value (95%CIs in parentheses) is for a combination of females 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

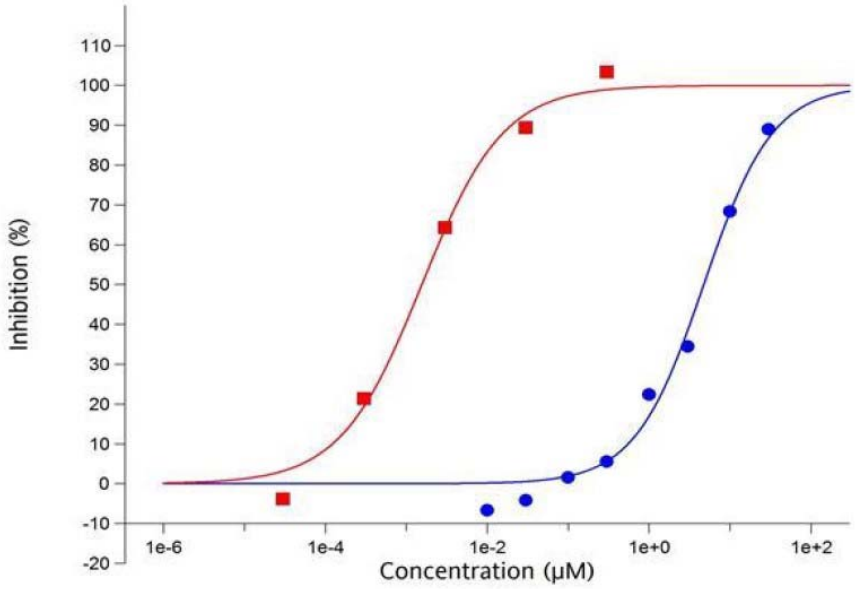
Morphine	First: **8.49 (4.73 — 23.6)** Reassessment: **9.81 (5.45 — 27.9)**	First: 1.81 (1.15 — 2.88) Reassessment: 1.16 (0.737 — 1.82)	First: 1.01 (0.822 — 1.25) Reassessment: 1.07 (0.860 — 1.32)
Morphine (females)	First: **4.97 (2.48 — 13.0)** Reassessment: **4.43 (2.55 — 8.03)**	First: 1.33 (0.428 — 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)



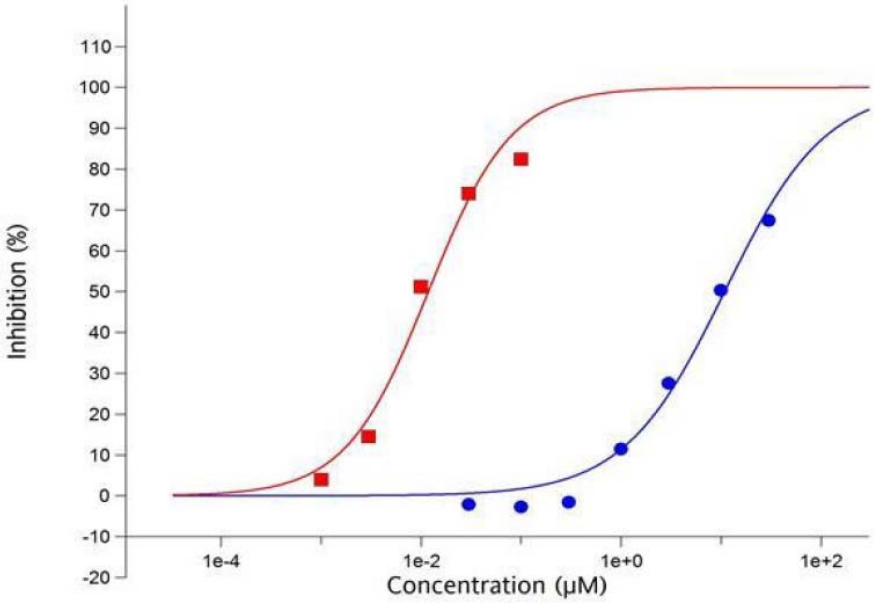
Supplemental Figure 1. Concentration-effect curves of mitragynine in stimulating [^{35}S]GTPγS binding in CHO cell lines stably expressing the human delta-, kappa-, and mu-opioid receptors from Eurofins Cerep (Celle l'Evescault, France). The EC_{50} values of the reference agonists DPDPE, U69,593, and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



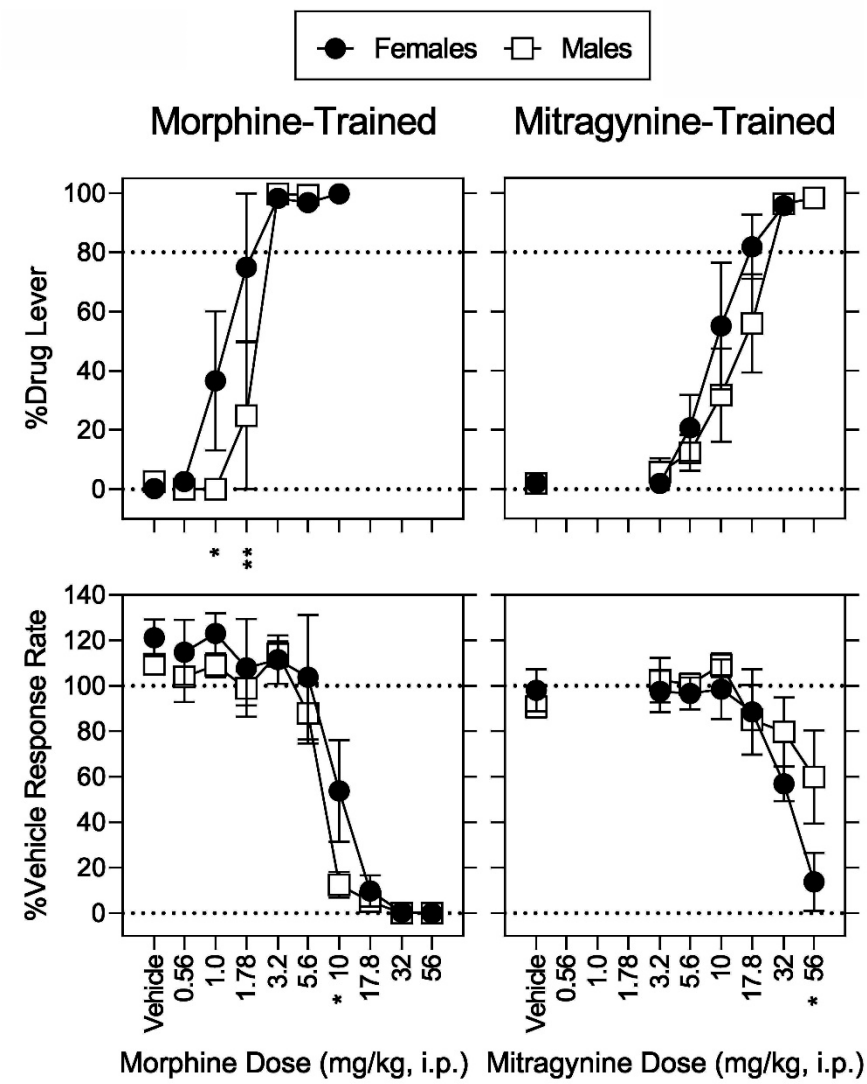
Supplemental Figure 2. Antagonism of DPDPE agonist effects (unspecified from Eurofins Cerep) by mitragynine (blue circles) and naltrindole (red squares) at the delta-opioid receptor using the [^{35}S]GTPγS binding from Eurofins Cerep (Celle l'Evescault, France). The IC_{50} values of mitragynine and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



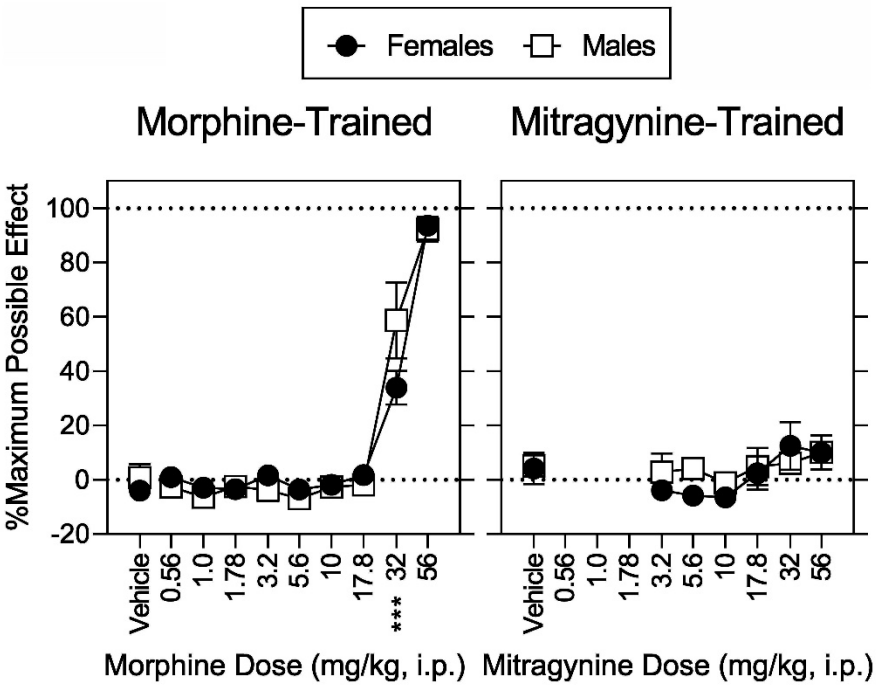
Supplemental Figure 3. Antagonism of U69,593 agonist effects (unspecified from Eurofins Cerep) by mitragynine (blue circles) and nor-binaltorphimine (red squares) at the kappa-opioid receptor using the [^{35}S]GTPγS binding from Eurofins Cerep (Celle l'Evescault, France). The IC_{50} of mitragynine and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



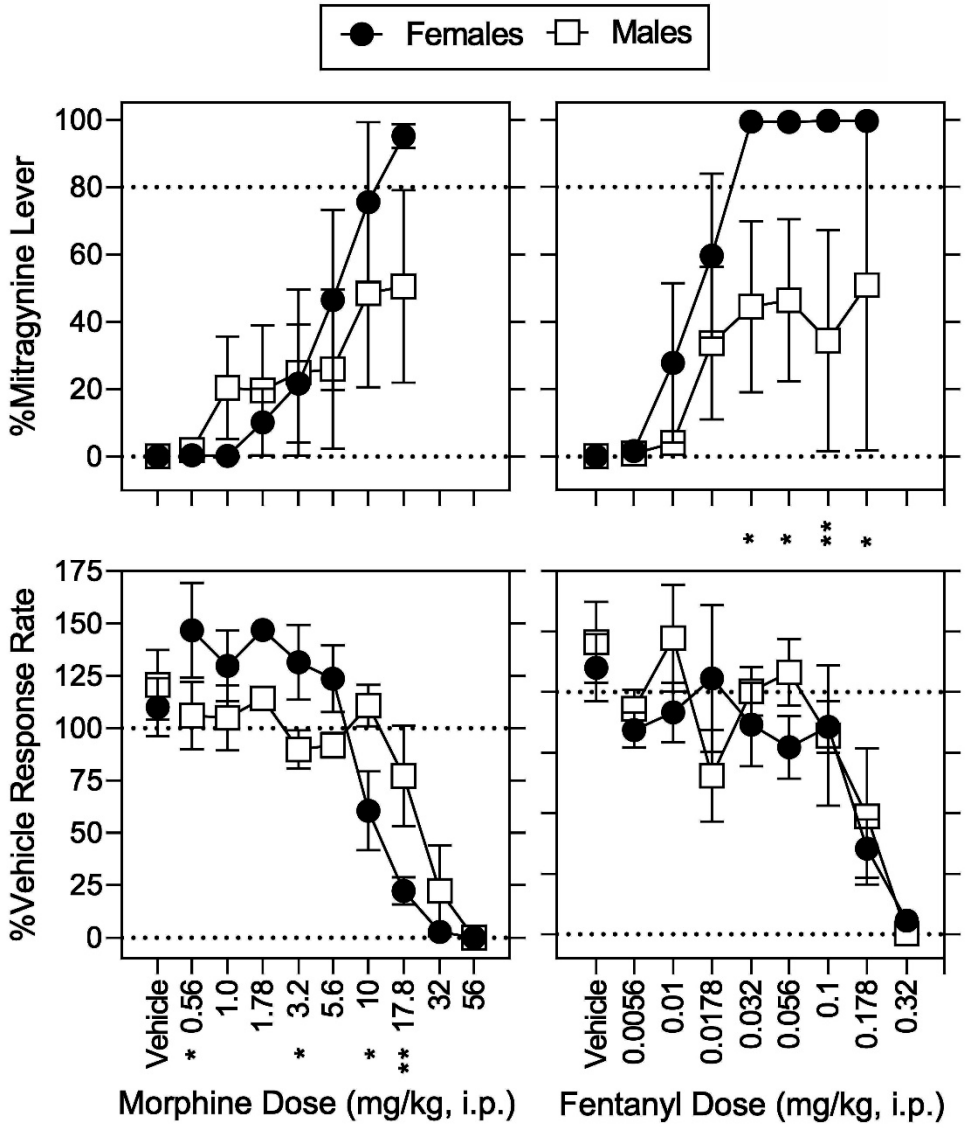
Supplemental Figure 4. Antagonism of DAMGO agonist effects (unspecified from Eurofins Cerep) by mitragynine (blue circles) and naltrexone (red squares) at the MOR using the [^{35}S]GTPγS binding from Eurofins Cerep (Celle l'Evescault, France). The IC_{50} values of mitragynine and naltrexone were 10.8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