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Supplementary Information for

**Positive allosteric modulation of the mu-opioid receptor produces analgesia with reduced side-effects**

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Supplementary Text Information
Figures S1 to S6

## Materials and Methods

### Drugs and Chemicals

R(-)-methadone HCl, morphine sulfate, and naloxone HCl were obtained from the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD, USA). BMS-986122 (2-(3-Bromo-4-methoxyphenyl)-3-[(4-chlorophenyl)sulfonyl]-thiazolidine) and RB-101 (12,13; N-[(R, S)-2-benzyl-3-[(S)(2-amino-4-methyl-thio)-butyldithio]-1-oxopropyl]-l-phenylalanine benzyl ester) were obtained or synthesized as previously described (1, 2). β-funaltrexamine HCl and methionine-enkephalin were purchased from Sigma-Aldrich. GTPγ$^{35}$S was purchased from PerkinElmer Life Sciences. All other chemicals, unless otherwise specified, were purchased as analytical grade from Sigma-Aldrich.

### In vitro assays

**[$^{35}$S]GTPγS binding assay.** Assays were performed in membrane homogenates from CHO cells expressing hMOR (kindly provided by Dr. L Toll, Department of Biomedical Science, Florida Atlantic University), from mouse brain (minus olfactory bulb and cerebellum) or from 1 mm sections of midbrain containing the PAG. Homogenates were prepared ice cold 50 mM Tris base, pH 7.4, as previously described (3). Membranes (10 μg protein) were pre-incubated for 10 min at 25°C with various concentrations of opioid or PAM, or both concurrently, in [$^{35}$S]GTPγS-binding buffer (50 mM Tris base [pH 7.4], 5 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 100 uM GDP, and 0.4 U/mL adenosine deaminase). After pre-incubation, 0.1 nM [$^{35}$S]GTPγS was added and reactions were further incubated for 90 min at 25°C. Reactions were stopped by rapid filtration through a Brandel MLR-24 harvested (Brandel, Gaithersburg, Maryland, USA), washed (50 mM Tris base [pH 7.4], 5 mM $MgCl_2$, and 100 mM NaCl), and bound radioligand collected on GF/C

filtermats (Whatman, Kent, UK). Bound radioactivity was measured using a Wallac 1450 MicroBeta counter (PerkinElmer, Waltham, Massachusetts, USA).

**β-arrestin 2 Recruitment.** Determined using commercially available PathHunter ® kit from DiscoverX (Freemont CA). Pathunter® CHO-hMOR β-Arrestin cells were plated at a density of 5,000 cells per well in 384-well white polystyrene cell culture plates 24 h prior to addition of MOR ligand and/or BMS-986122 for 60 min at $37^0$C. Cells were then treated with β-galactosidase substrate incubated for 60 min at room temp and luminescence detected using an Envision Plate Reader (Perkin Elmer).

**Brain Slice Electrophysiological Recordings.** Coronal slices (220 μm) of brains from male Sprague-Dawley rats (postnatal day >30) containing ventrolateral periaqueductal gray (vlPAG) were cut and maintained at 32°C in physiological saline equilibrated with 95% $O_2$ and 5% $CO_2$ and then transferred to a small chamber (32 °C) for electrophysiological recording as described (4). The extracellular solution contained 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 2.4 $CaCl_2$, 1.2 mM $NaH_2PO_4$, 21.4 mM $NaHCO_3$, and 11.1 mM glucose (pH 7.35). vlPAG neurons were visualized with infrared Nomarski optics and whole-cell patch clamp recordings were made with patch electrodes (2-5 MOhms) with an internal solution containing 130 mM CsCl, 10 mM HEPES, 1.1 mM EGTA, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 4 mM MgATP, 1 mM NaGTP, and 0.3% biocytin (pH 7.4). Series resistance (<12 MOhms) was compensated by 80% and continuously monitored throughout the experiment. Liquid junction potentials of 5 mV were corrected. Data were acquired with Axograph X (Sydney, Australia) software. Spontaneous miniature inhibitory post-synaptic currents (mIPSCs) were obtained in the presence of tetrodotoxin (500 nM) and NBQX (5 μM) filtered at 2 kHz and sampled at 5 kHz. Events were detected using Axograph by selecting events that exceeded a preset threshold (set to 10-20 pA) and fit the criteria: 10-90%

rise time (0 and 2 ms) and half-width (> 3 ms). Events were verified individually, and frequency was determined over two-minute epochs after equilibration of each drug

**Behavioral assays**

Animal numbers per group for behavioral tests are indicated in the figure legends. All behavioral assays included controls (vehicle) run at the same time. The i.c.v. injections were not blinded, but these assays were performed by four different individuals, each trained to observe the same endpoint. All assays involving peripheral administration of BMS-986122 were performed blinded to drug or vehicle (numbered not named) or pretreatment condition.

**Hot-plate test**. This was used to measure supraspinally-mediated antinociception following intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) administration of compounds as previously described (5). Briefly, mice were placed on a hot plate analgesia meter (Columbus Instruments, Columbus, Ohio, USA) maintained at 52 °C and contained within a 15 cm high plastic square. The latency to lick or shake the forepaw(s) or hindpaw(s) or jump was measured. A cutoff time of 60 sec was used to prevent tissue damage. For all experiments, baseline latencies were recorded prior to drug administration. BMS-986122 was co-administered with orthosteric drugs when administered i.c.v. BMS-986122 was given as a 30 min pretreatment when orthosteric drugs were administered i.p. For the antagonist experiments, naloxone was administered 30 min prior to BMS-986122 administration.

**Warm water tail withdrawal.** The warm water tail withdrawal test was used as a measure of spinally-mediated nociception as previously described (5). Briefly, mice were placed into a plastic restrainer. The distal 2-3 cm of the tail was placed into a water bath maintained at 55 °C. The latency to withdraw the tail from the water was measured. A cutoff time of 15 sec was used to prevent tissue damage. For all experiments, baseline latencies were recorded prior to drug

administration. BMS-986122 was given as a 30 min pretreatment when orthosteric drugs were administered i.p. For the antagonist experiments, naloxone was administered 30 min prior to BMS-986122 administration. For repeated injection studies, after baseline thresholds were obtained on the on the afternoon of Day 1 (3:00 pm), animals were injected with BMS-986122, morphine, or vehicle and tested 30 min after injection. On Day 2, animals received an injection of BMS-986122, morphine or vehicle in the morning (9:00 am) but were not tested. Six hours later (3:00 pm), animals were injected with BMS-986122, morphine, or vehicle and tested 30 mins later.

**Swim stress-induced antinociception**. Methods were adapted from published literature (6). Briefly, after baseline hot plate latencies were obtained animals were injected with BMS-986122 i.c.v. Ten min after drug administration, mice were placed in a glass cylinder (18 cm tall x 14 cm diameter) filled with 25 °C water to a depth of 14 cm. Following 15 min of swim stress, mice were placed under a heat lamp to dry for 5 min. Mice were then re-assessed for hot-plate latencies.

**Pain-depressed nesting.** This was performed as described (7). Briefly, a cotton nestlet was cut into six equal pieces and placed along the walls of a cage with fresh bedding. Each piece occupies one zone. Animals were placed in the cage and allowed to nest for 120 min. The amount of zones cleared (i.e., where the animal removes the piece of nestlet and uses it to construct a nest) was counted after each 15 min period. The following day, animals were administered BMS-986122, morphine, or vehicle and returned to their home cage. 30 min later, animals were injected with 0.6% acetic acid to induce visceral pain. Immediately after acid injection, animals were placed in a cage with fresh bedding and six nestlet pieces. Animals were allowed to nest for 120 min and number of zones cleared counted every 15 min.

**Inflammatory pain testing using Complete Freund's Adjuvant (CFA) or carrageenan.**

Inflammatory pain was induced *via* unilateral intraplantar (i.pl.) administration of either 2.5% carrageenan (20 μL) or Complete Freund's Adjuvant (CFA; 5 μL) (both purchased from Sigma-Aldrich, St. Louis, Missouri, USA) into the right hindpaw. Animals were placed on an elevated platform and von Frey filaments (North Coast Medical, Morgan Hill, California, USA) were used to assess mechanical allodynia in the inflamed paw using a modified up-down method(8). For the time course studies (Figure 5C). Baseline thresholds were obtained in the von Frey test. On the afternoon of day 1, animals were injected with CFA into the right hindpaw and returned to their home cage. Twenty-four hours after CFA injection, animals were injected with BMS-986122, morphine, or vehicle and mechanical withdrawal thresholds were obtained from the right hindpaw 30 min after injection. Forty-eight hours after CFA injection animals received an injection of drug in the morning but were not tested. Six hours later, animals were injected with BMS-986122, morphine, or vehicle and re-tested. This was repeated 72 hours after CFA injection.

**Conditioned Place Preference (CPP).** Mice were placed randomly assigned to one chamber of the apparatus on day 1 and allowed to freely explore for 30 min. Time spent in each chamber was recorded. Mice were randomly assigned to be conditioned with morphine, BMS-986122, or vehicle in one of two chambers. During conditioning, mice were given a vehicle injection (i.p.) and immediately placed in the vehicle-paired chamber for 30 min; 6 h later, mice were given an injection of morphine, BMS-986122, or vehicle (i.p.) and immediately placed in the drug-paired chamber for 30 min. This conditioning was repeated for 5 consecutive days. After conditioning, mice were randomly placed in either compartment and allowed to roam freely for 30 min. No injection was given on test day. Time spent in each chamber was recorded. CPP scores were

calculated as the difference between time spent on the drug-paired side on test day compared to the time spent on the future drug-paired side on Day 1.

**Constipation.** Mice were injected i.p. with morphine, BMS-986122, or vehicle and immediately placed in individual cages with a mesh bottom. The number of fecal boli deposited were counted each hour for two hours.

**Respiratory Depression.** Respiration rates were measured using the respiratory monitor component of the Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) as described previously (9). Freely moving mice were habituated in closed, sealed individual boxes (23.5 cm x 11/5 cm x 13 cm) for 60 min before testing. Mice were then pretreated with vehicle or BMS-986122 (10mg/kg, i.p.). Five minutes later, mice were confined to the testing apparatus for 120 min. Using a pressure transducer built into the sealed CLAMS box, the respiration frequency of each occupant mouse was collected sequentially every 30 seconds and grouped into 5 minute bins until the completion of the test.

**<u>Plasma and brain levels of BMS-986122</u>**

Determined by the Pharmacokinetics and Mass Spectrometry Core in the College of Pharmacy, University of Michigan as follows**.** Male and female 129S1/SvlmJ mice were treated with BMS-986122 (10mg/kg i.p) in the same vehicle used for the behavioral experiments. Mice were anesthetized with isoflurane and blood samples were collected at 1h post-injection from the orbital sinus into heparinized tubes, followed by centrifugation at 3000*g* for 10 min at 4 °C to obtain plasma. Mice were then immediately euthanized by cervical dislocation, brains collected, washed with PBS and homogenized in PBS with 20% acetonitrile. All samples were immediately frozen using liquid nitrogen, and stored at −80 °C. BMS-986122 was extracted with acetonitrile and subjected to LC-MS/MS analysis using a Shimadzu HPLC system with a Waters XBridge-C18 column (5 cm × 2.1 mm, 3.5 µm) and mobile phases of 0.1% formic acid in purified water (A) and

0.1% formic acid in acetonitrile (B) with a flow rate of 0.5mL/min. BMS-986122 was detected using an AB Sciex QTrap 5500 mass spectrometer equipped with an electrospray ionization source (Applied Biosystems, Toronto, Canada) in the positive-ion multiple reaction monitoring (MRM) mode. Values were determined from a standard curve of BMS-986122 (2.5 - 10,000 ng/mL) in blank plasma or brain samples.

Figures

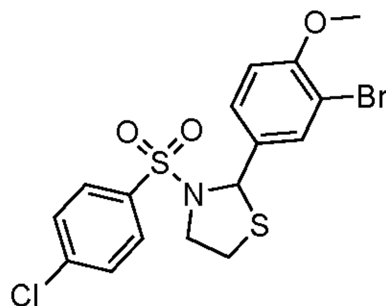


Fig. S1. Structure of BMS-986122: (2-(3-Bromo-4-methoxyphenyl)-3-[(4-chlorophenyl)sulfonyl]-thiazolidine).

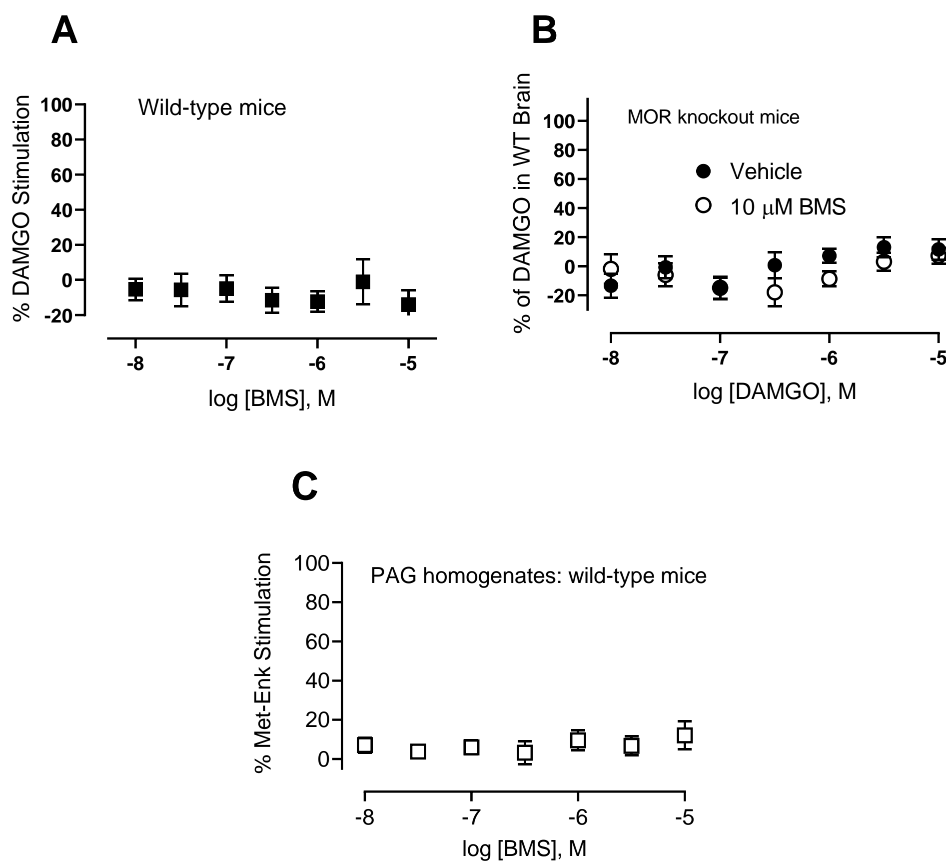


Fig. S2. A) Effect of BMS-986122 (BMS) on stimulation of [$^{35}$S]GTP$\gamma$S binding in whole brain homogenates from wild-type C57BL/6 mice. B) Effect of DAMGO in the absence (vehicle) and presence (BMS) of BMS-986122 on stimulation of [$^{35}$S]GTP$\gamma$S binding in whole brain homogenates from MOR knockout mice on a C57BL/6 background. C) Effect of BMS-986122 (BMS) on [$^{35}$S]GTP$\gamma$S binding in PAG homogenates from wild-type C57BL/6 mice.

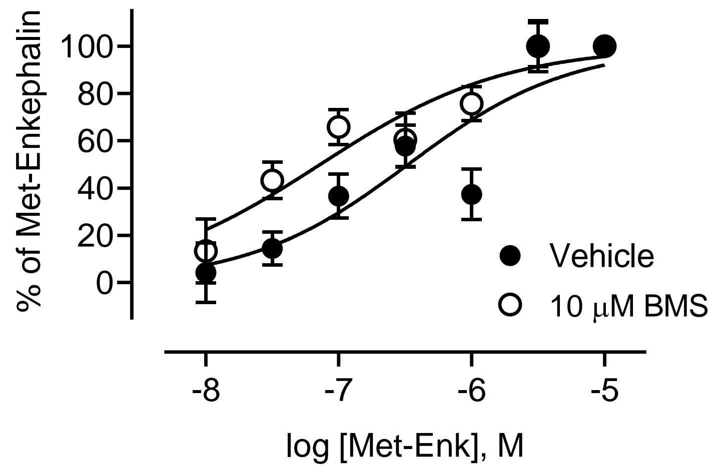


Fig. S3. Effect of BMS-986122 (BMS) on Met-Enk-mediated stimulation of [$^{35}$S]GTPγS binding in whole brain homogenates from wild-type C57BL/6 mice. The $EC_{50}$ value for Met-Enk is 335 (176-623) nM which shifts to 72.8 (36.6-138) nM in the presence of 10μM BMS 986122, a shift of 4.6-fold. The Hill Slopes are 0.72 (0.5-1.1) and 0.63 (0.42-0.94) respectively.

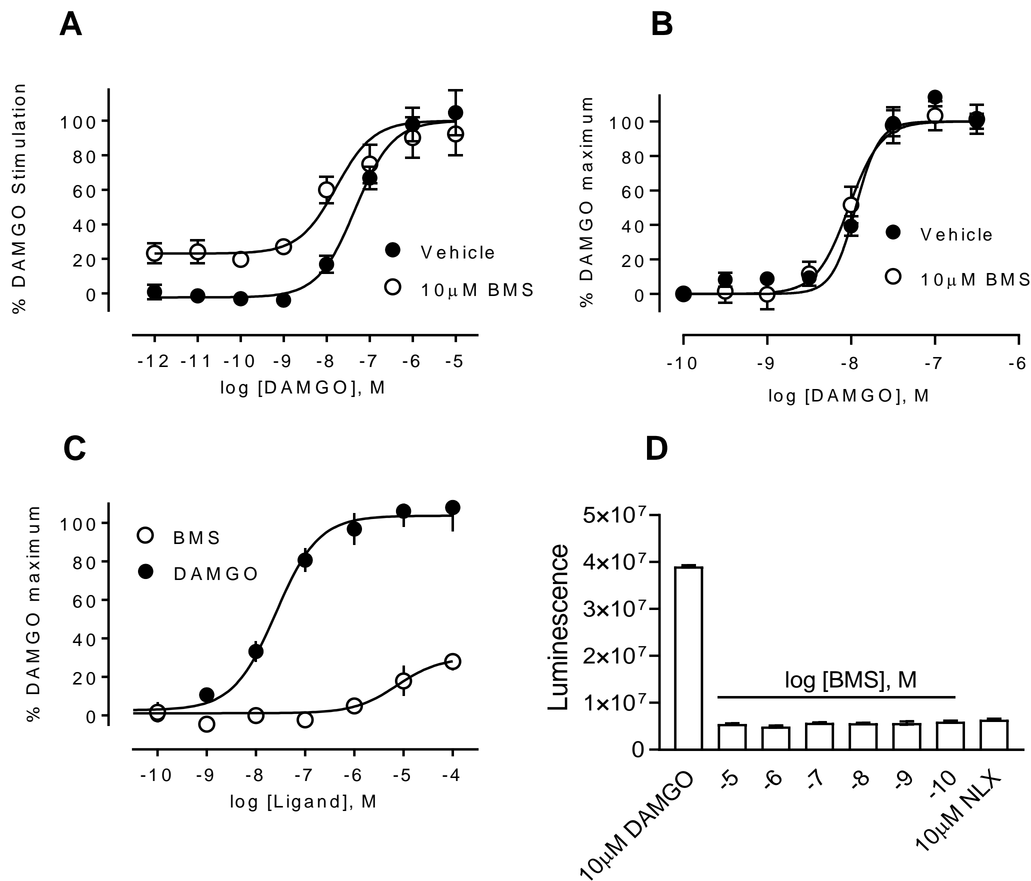


Fig. S4. A) DAMGO stimulation of [$^{35}$S]GTP$\gamma$S binding in membranes of CHO cells expressing hMOR. B) DAMGO recruitment of β-arrestin 2 in Pathhunter CHO cells expressing hMOR. C) BMS 986122 (BMS) effect on [$^{35}$S]GTP$\gamma$S binding in membranes of CHO cells expressing hMOR. D) Lack of effect of BMS 986122 (BMS) on recruitment of β-arrestin 2. Data points are means ± SEM from 3 separate experiments performed in duplicate.

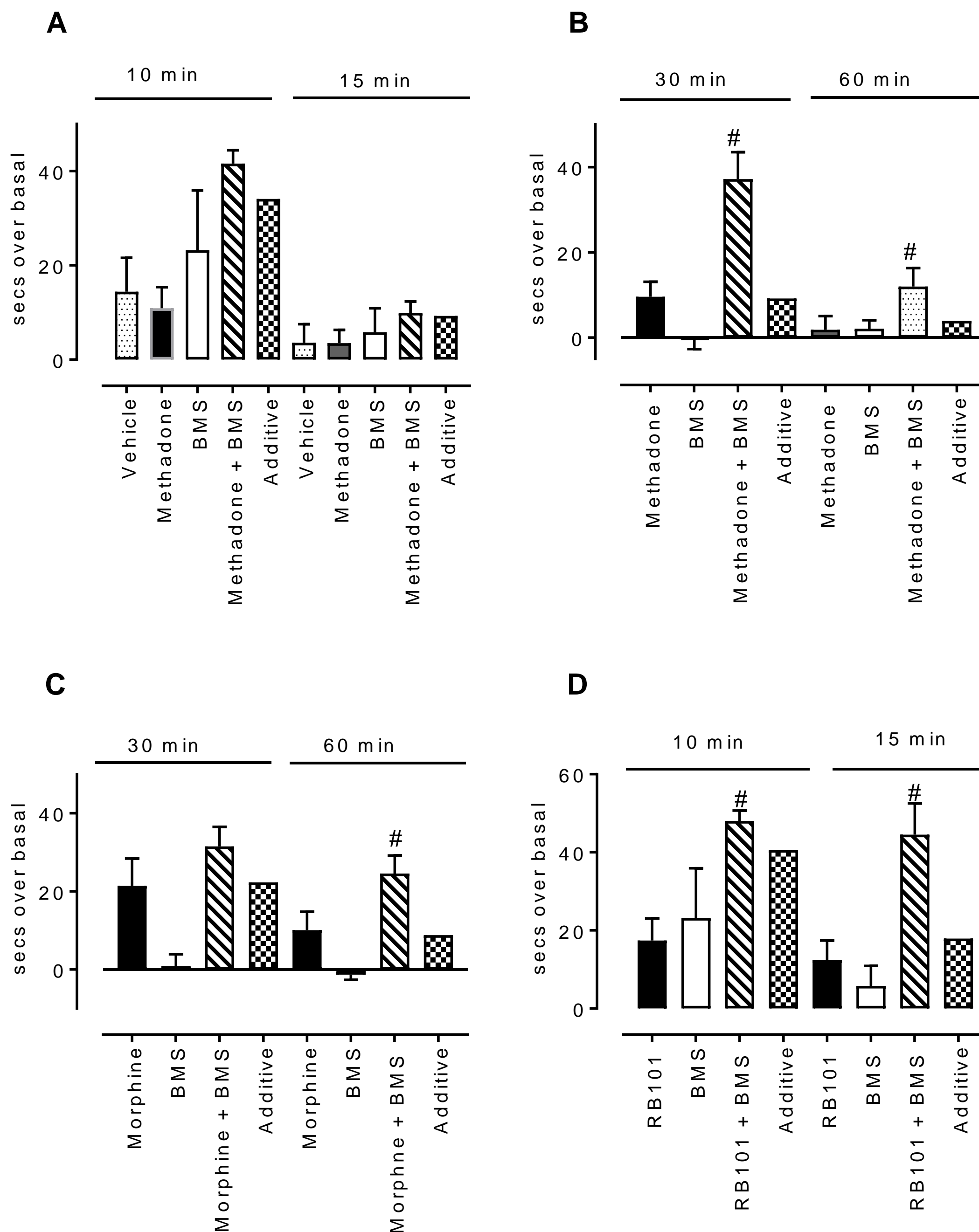


Figure S5. Effects of (A) methadone (icv), (B) methadone (i.p.), (B) morphine (i.p.) or (C) RB101 (i.c.v.) on hot-plate latency in C57BL/6 mice in the presence or absence of BMS-986122 (BMS; i.c.v). Data are derived from Fig. 2 and presented as latency – basal values ± S.D in secs from 6 mice in each group with each mouse acting as its own basal control. Times refer to post-injection interval. Additive columns refer to addition of the group means from the opioid or RB101 alone and BMS-986122 alone treatment groups. # Co-treatment values are greater than the corresponding additive value by more than 2 SDs, indicating synergy.

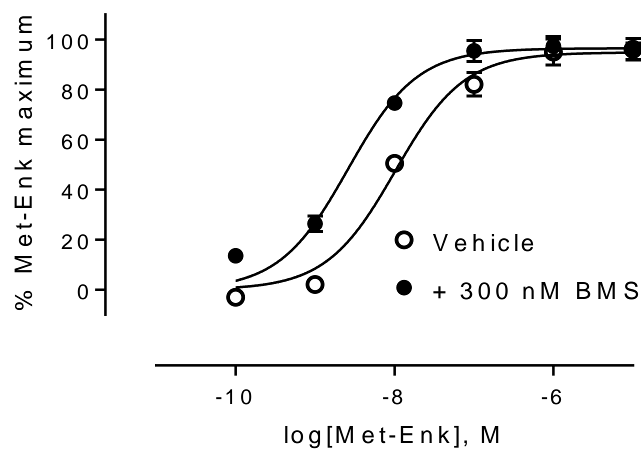


Figure S6. Effect of 300nM BMS-986122 (BMS) on Met-Enk-mediated stimulation of [$^{35}$S]GTPγS binding in membranes of CHO cells expressing hMOR. The $EC_{50}$ value for Met-Enk in the absence of BMS-986122 is 10.2 (7.3-14.2) nM and in the presence of BMS-986122 is 2.6 (1.9-3.6) nM. Data points are means ± SEM from 3 separate experiments performed in duplicate.

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