JPET Fast Forward. Published on September 6, 2019 as DOI: 10.1124/jpet.118.255661
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Title Page

# Preclinical testing of nalfurafine as an opioid-sparing adjuvant that potentiates analgesia by the mu opioid receptor-targeting agonist morphine*

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Running Title Page

**Running title:** Testing G protein biased KOR agonists as morphine add-ons

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**Number of text pages:** 25

**Number of tables:** 0

**Number of figures:** 8 (+ 1 supplementary figure)

**Number of references:** 91

**Number of words in Abstract:** 246

**Number of words in Introduction:** 752

**Number of words in Discussion:** 2,264

**Nonstandard abbreviations:** ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; CPA, conditioned place aversion; CPP, conditioned place preference; EOM salvinorin B, ethoxymethyl ether salvinorin B; KOR, kappa opioid receptor; MOR, mu opioid receptor; OUD, opioid use disorder.

**Recommended section assignment:** Behavioral Pharmacology

**Abstract:**

Mu opioid receptor (MOR)-targeting analgesics are efficacious pain treatments, but notorious for their abuse potential. In preclinical animal models, co-administration of traditional kappa opioid receptor (KOR)-targeting agonists with MOR-targeting analgesics can decrease reward and potentiate analgesia. However, traditional KOR-targeting agonists are well known for inducing anti-therapeutic side effects (psychotomimesis, depression, anxiety, dysphoria). Recent data suggest that some functionally selective, or biased, KOR-targeting agonists might retain the therapeutic effects of KOR activation without inducing undesirable side effects. Nalfurafine, used safely in Japan since 2009 for uremic pruritus, is one such functionally selective KOR-targeting agonist. Here, we quantify the bias of nalfurafine and several other KOR agonists relative to an unbiased reference standard (U50,488), and show that nalfurafine and EOM salvinorin B demonstrate marked G protein signaling bias. While nalfurafine (0.015 mg/kg) and EOM salvinorin B (1 mg/kg) produced spinal anti-nociception equivalent to 5 mg/kg U50,488, only nalfurafine significantly enhanced the supraspinal analgesic effect of 5 mg/kg morphine. In addition, 0.015 mg/kg nalfurafine did not produce significant conditioned place aversion (CPA), yet retained the ability to reduce morphine-induced conditioned place preference (CPP) in C57BL/6J mice. Nalfurafine and EOM salvinorin B each produced robust inhibition of both spontaneous and morphine-stimulated locomotor behavior, suggesting a persistence of sedative effects when co-administered with morphine. Taken together, these findings suggest that nalfurafine produces analgesic augmentation, while also reducing opioid-induced reward with less risk of dysphoria. Thus, adjuvant administration of G protein-biased KOR agonists like nalfurafine may be beneficial in enhancing the therapeutic potential of MOR-targeting analgesics, such as morphine.

## Introduction

Opioid use disorder (OUD) is an American health crisis (Manchikanti et al., 2012; Koh, 2015; Volkow and Collins, 2017). Use of prescription opioids, such as morphine and oxycodone, has contributed significantly to the OUD problem (Jones, 2013). Morphine, the prototypical opioid analgesic, is a mainstay for treating moderate-to-severe pain, as may occur post-operatively. While prescription opioids are effective painkillers, currently there exists no facile way to use them without the prospect of inducing addiction. One plausible solution is to use morphine (and other MOR-targeting analgesics) in a way that increases drug-induced analgesia, thereby requiring lower doses (an approach known as dose sparing), or in a way that reduces addictive potential. In this vein, if the anti-nociceptive potential of MOR-targeting analgesics could be enhanced *via* a dose-sparing, anti-addictive adjuvant that does not induce therapeutically limiting side effects, then their use in treating pain could be continued.

Kappa opioid receptor (KOR)-targeting agonists, when given with MOR-targeting analgesics, not only produce additive analgesia (Sutters et al., 1990; Negus et al., 2008; Briggs and Rech, 2009), reduced tolerance (He and Lee, 1997; Khotib et al., 2004), and reduced respiratory depression (Verborgh et al., 1997) but also can reduce the rewarding properties of MOR-targeting analgesics (Kuzmin et al., 1997; Tsuji et al., 2001; Tao et al., 2006). However, clinical use of some KOR agonists, such as ketocyclazocine (Kumor et al., 1986) or salvinorin A (MacLean et al., 2013), has largely failed due to poor tolerability. In rats and mice, traditional KOR agonists produce anxiety- and depression-associated behaviors (Carlezon et al., 2006; Bruchas et al., 2009; Ehrich et al., 2015), as well as locomotor suppression (Kunihara et al., 1993; Narita et al., 1993; Ehrich et al., 2015; Brust et al., 2016). In humans, the highly selective KOR agonist salvinorin A produces similar therapeutically limiting effects (MacLean et al., 2013; Maqueda et al., 2015; Johnson et al., 2016).

Thus, it is not surprising that initial enthusiasm for KOR agonists as adjuvants to MOR-targeting analgesics was reduced by early preclinical and clinical findings using mixed MOR/KOR agonists. For example, while nalbuphine studies in rats demonstrated reduced conditioned place preference (CPP) to morphine and enhanced morphine-induced analgesia (Tao et al., 2006), human studies demonstrated dose-dependent increases in dysphoria (Jasinski and Mansky, 1972; Preston et al., 1989; Walsh and Babalonis, 2017). Similarly, pentazocine and butorphanol also induce the classic dysphoric effects of traditional KOR-targeting agonists in humans, especially at higher doses (Preston et al., 1987; Zacny et al., 1994; Greenwald and Stitzer, 1998; Walsh et al., 2008; Walsh and Babalonis, 2017), limiting their therapeutic potential.

Recent efforts to develop non-addictive opioids however, have renewed interest in KOR ligands with specific emphasis on developing functionally selective (or biased) KOR agonists (Urban et al., 2007; Rankovic et al., 2016). G protein-biased KOR agonists demonstrate reduced potencies for signaling through the GRK/β-arrestin pathway (Bohn and Aube, 2017). While G protein-dependent signaling through KOR is well established as producing the primary therapeutic outcome of analgesia (Chavkin, 2011; Brust et al., 2016; Schattauer et al., 2017), the role of GRK/β-arrestin signaling in producing dysphoria downstream of KOR activation is less clear. Some have shown that aversive behavior toward KOR agonists requires GRK3 (and p38 MAPK) activation (Bruchas et al., 2007; Chavkin et al., 2014; Ehrich et al., 2015), while others have observed little effect of *Arrb2* (β-arrestin-2) genetic ablation on the aversion produced by a variety of KOR agonists (White et al., 2015). Conversely, recent work with triazole 1.1 (Brust et al., 2016) and nalfurafine (Liu et al., 2018) suggests that these G protein-biased KOR agonists may lack dysphoric effects at doses that produce analgesia, supporting the role of β-arrestin signaling in the dysphoric effects of traditional KOR agonists. G protein-biased KOR agonists may, thus, present

a strategy for reducing the rewarding properties of MOR-targeting analgesics while avoiding the pitfalls of traditional KOR agonists. This notion is supported by a recent preclinical study in rats (Townsend et al., 2017a) showing that nalfurafine reduces intravenous self-administration of the MOR-targeting analgesic oxycodone while also potentiating its analgesic effect.

Nalfurafine is a G protein functionally selective KOR-targeting agonist with high translational potential given its safe use in Japan since 2009 to treat uremic pruritis and, importantly, the drug does not produce psychotomimesis (Inui, 2015). Therefore, we aimed to assess the therapeutic efficacy of nalfurafine in both potentiating the analgesic effect and reducing the rewarding properties of the MOR-targeting analgesic morphine, while also assessing the biased signaling properties of nalfurafine *in vitro* as compared with other reported G protein-biased KOR agonists.

**Methods:**

*Animal Subjects*

All experiments were conducted using male and female C57BL/6J mice (RRID: IMSR_JAX:000664) between 8-14 weeks of age and 17-35 g; the sexes were used in similar proportions across all experiments. Original breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were group housed in an AAALAC-accredited facility, with free access to food and water. All procedures were carried out in accordance with the National Research Council's *Guide to the Care and Use of Laboratory Animals (Eighth Edition)*, and were approved by West Virginia University's Institutional Animal Care and Use Committee.

*Study Design*

Mice were randomly assigned to treatment conditions, after being balanced for sex. Mice were acclimated to the test room for at least 30 min prior to any study. All mice were used for a single experimental assay. Sample size for each experiment was determined by power analysis using effect sizes derived from literature, power value of 0.8, and an alpha value of 0.05. All experiments were done by personnel who were blinded to treatment conditions.

*Drugs*

(±) U50,488, GR 89696, and ICI 199,441 were all purchased from Tocris Biosciences (Minneapolis, MN). Nalfurafine hydrochloride was purchased from Medchem Express (Monmouth Junction, NJ). Morphine sulfate salt pentahydrate was purchased from Sigma Aldrich (St. Louis, MO). Salvinorin A, ethoxymethyl ether salvinorin B (EOM salvinorin B), and triazole 1.1 were prepared as previously described (Zhou et al., 2013; Ewald et al., 2017). All drugs were dissolved in 100% DMSO and diluted in saline to the desired concentration (no DMSO concentration exceeded 5% v/v final). In drug combination experiments, nalfurafine was injected subcutaneously (sc) as described in the limited literature available regarding this compound's use in mice (Endoh et al., 1999; Tsuji et al., 2001; Liu et al., 2018), while U50,488, EOM salvinorin B and morphine were delivered by intraperitoneal (ip) injection as commonly described (Rada et al., 1996; McLaughlin et al., 2006; Koo et al., 2012; Muschamp et al., 2012; Laman-Maharg et al., 2017; Robinson et al., 2017). Dose ranges for nalfurafine, EOM salvinorin B and U50,488 were chosen based upon existing conditioned place preference and tail immersion data in the literature (Tsuji et al., 2001; Land et al., 2009; Ehrich et al., 2015). The morphine dose was chosen to

produce consistent place preference while allowing for high sensitivity to detect potentiation in the hot plate assay of analgesia (Mueller et al., 2002; Raehal and Bohn, 2011; Ewald et al., 2017)

*Measurement of Signaling Bias*

Quantification of G protein signaling dependent changes in intracellular cyclic AMP (cAMP) levels was conducted using a GloSensor luciferase-based assay in HEK293T cells transiently transfected with 5 μg of KOR expression vector DNA (3xHA-hKOR; www.cDNA.org) and 5 μg of pGloSensor-22F cAMP biosensor expression vector DNA (Promega, Madison, WI). Quantification of β-arrestin recruitment was conducted using the luciferase-based TANGO assay (Barnea et al., 2008; Allen et al., 2011) in HTLA cells (generously provided by Dr. Gilad Barnea) transiently transfected with 5 μg of KOR-V2-TEV-tTA expression vector DNA (Addgene plasmid #66462 donated by Dr. Bryan Roth). The appropriate masses of expression vector DNAs transfected was empirically determined prior to acquisition of the data contained herein. Transfections were performed in 10-cm dishes using the calcium phosphate-based method (Jordan et al., 1996). Culture media was replaced 16 hrs after transfection, and cells then plated on 96-well plates approximately 24 hrs after transfection. All cells were serum-starved with 1% dialyzed serum-containing medium for 8-16 hrs before assaying. For assays of cAMP inhibition, medium was replaced with GloSesnor detection reagent dissolved in HEPES (20 mM)-buffered Hank's balanced saline solution, and cells were incubated for 2 hrs at room temperature. KOR-selective agonists were applied for 15 min prior to a 15-min stimulation with 100 nM isoproterenol (to induce endogenous β-adrenergic receptor-mediated and $G\alpha_s$-dependent production of cAMP) and subsequent quantification of luminescence. For TANGO assays of β-arrestin recruitment, drug stimulation was performed overnight in 1% dialyzed serum-containing medium. Medium was

removed the following morning, the cells lysed with BrightGlo reagent (Promega), and luminescence quantified using a FlexStation 3 multi-mode plate reader (Molecular Devices).

*Calculation of Bias Factor*

Bias factors were calculated using the method previously described (Kenakin, 2017). Briefly, $Log(E_{max}/EC_{50})$ values for both GloSensor and TANGO assays were separately generated for each ligand using potency ($EC_{50}$) and maximal efficacy ($E_{max}$) values obtained from these assays. $\mathbf{\Delta}Log(E_{max}/EC_{50})$ was calculated in each assay as the difference in $Log(E_{max}/EC_{50})$ for each ligand from the reference ligand (the unbiased KOR agonist U50,488). $\mathbf{\Delta\Delta}Log(E_{max}/EC_{50})$ was then calculated as the difference in $\mathbf{\Delta}Log(E_{max}/EC_{50})$ between GloSensor and TANGO assays for each ligand. The 95% confidence interval (CI) for each bias factor was calculated using the $E_{max}$ and $EC_{50}$ values generated in individual experiments for each ligand in each assay.

*Warm Water Tail Withdrawal*

The warm water tail withdrawal test was conducted as described previously (Schattauer et al., 2017). Briefly, mice were gently wrapped in cloth before immersing the distal 2 cm of the tail in 55°C water and recording the latency for the mouse to withdraw its tail from the water. Baseline latencies were recorded 30 min after administration of vehicle. Mice were then injected with drug and latencies were recorded after 30 min. A maximum cutoff of 10 seconds was used to avoid tissue damage. As the relevant comparison is to the analgesic effect of morphine alone, data are presented as latency to tail-withdrawal in seconds (Figure 4) as well as percent maximum possible effect (%MPE, Figure S1) following the equation: %MPE = (latency - baseline) / (10 sec - baseline) * 100%).

*Hot Plate Analgesia Test*

To assess integrated pain perception, the hot plate test of nociception was performed as described previously (Raehal and Bohn, 2011) with minor modification. Nociceptive latency after vehicle injection was measured on Day 1. On Day 2, mice were injected with drug and response latencies were measured after 30 min. Individual mice were placed onto a 53ºC hot plate (IITC Life Science Inc.; Woodland Hills, CA) inside of a clear, open-bottom acrylic cylinder. A maximum cutoff of 30 s was used to avoid tissue damage. Time until the first nociceptive sign (*i.e.*, licking of the hind paw, flicking of the hind paw, or jumping) was recorded by a trained observer to the nearest 0.1 sec and reported as percent maximum possible effect (%MPE = (latency - baseline) / (30 sec - baseline) * 100%). Data in the inset of Figure 5B were normalized to the average %MPE of 5 mg/kg morphine.

*Locomotion*

Locomotor activity was assessed using a 16 X 16 open-field infrared photobeam activity system (PAS) from San Diego Instruments (San Diego, CA), as described previously (Gross et al., 2018). Briefly, locomotion was quantified as total beam breaks in the *x* and *y* coordinates in five minute bins. Spontaneous locomotion was assessed by administering drug or vehicle immediately before placing the mouse in the locomotor chamber for 60 min. Morphine-induced locomotion was assessed in a three-day procedure. On Day 1, mice were habituated to the locomotor chamber for 60 min. On Day 2, mice were habituated to the locomotor chamber for 30 min before receiving an injection of vehicle and returning to the chamber for an additional 60 min. On Day 3, mice were habituated to the locomotor chamber for 30 min before receiving an injection of either morphine with vehicle or morphine with KOR agonist, and returning to the chamber for an additional 60 min. Data plotted over time were normalized to the average vehicle locomotion in the first 5-min

bin for novelty-induced locomotion and to the average locomotion of each subject during the 30 mins prior to injection for morphine-suppressed locomotion. For total novelty-induced locomotion, data are normalized to the average total locomotor activity of the vehicle condition over the full 60-min trial. For total morphine-stimulated locomotion, data are normalized to the average total locomotor activity during the 60 minutes post vehicle injection on day 2.

*Conditioned Place Preference/Aversion*

CPP and CPA studies were conducted as described previously (Redila and Chavkin, 2008) with the following modifications. A two-chambered place preference apparatus with differential floor grating in the white and black chambers was used. Baseline preference was established on Day 1 by allowing the mouse free access to both chambers for 30 min (for U50,488 and EOM salvinorin B studies) or 50 min (for nalfurafine studies, given prior evidence of time-of-effect for this compound (Tsuji et al., 2001)) and recording the amount of time spent in each chamber. The conditioning phase occurred on days 2 and 3 in 30 min (U50,488, EOM salvinorin B) or 50 min (nalfurafine) sessions. Vehicle was administered in the AM session and drug in the PM session with a 4-hr interval between sessions. For CPP, drug was administered in the least-preferred chamber to override the initial preference of the mouse. Drug was administered in the most-preferred chamber for CPA. Preference was assessed on Day 4 by allowing the mouse free access to both chambers for 30 min (U50,488, EOM salvinorin B) or 50 (nalfurafine) min, and recording the amount of time spent in each chamber. ANY-maze video tracking software (Stoelting Co., Wood Dale, IL) was used to track movement and time spent in each chamber. Data are presented as the difference in time spent in the drug-paired chamber for each mouse. Data were analyzed by

two-way ANOVA with Dunnett's *post hoc* test to compare to vehicle controls and Holm-Sidak's *post hoc* test to compare across genotypes, each run using Prism 7 software (GraphPad).

*Rotarod*

Motor coordination was assessed using the rotational rod assay (Ugo Basile, Gemonio, Italy) as previously described (White et al., 2014) with minor adjustment. The rod was set to begin rotating at 4 rpm and accelerate to 40 rpm over the course of 5 min. Mice were trained on the rotarod on day 1 in two 5 min sessions. On day 2, the baseline latency to fall off the rod was determined and then mice were injected with either vehicle or drug. Mice were then assessed at 10, 30 and 60 min post injection. Data are reported as percent of baseline performance.

## Results

*Selection of nalfurafine and EOM salvinorin B as KOR biased agonists under consideration as possible morphine adjuvants*

Before launching our assessment of the efficacy of G protein-biased KOR agonists in reducing the addictive properties of morphine, we characterized the signaling properties of a variety of KOR-selective compounds to identify suitably functionally selective KOR agonists. Using the GloSensor-based cAMP assay to determine G protein signaling and the Tango assay to determine β-arrestin recruitment, we identified EOM salvinorin B (bias factor = 15.26, +95%CI = 29.95, -95%CI = 7.78) and nalfurafine (bias factor = 7.73, +95%CI = 15.16, -95%CI = 3.94) as strongly G protein-biased KOR agonists compared with U50,488 (Figure 1), the latter consistent with prior findings (Schattauer et al., 2017).

*Preclinical evaluation of EOM salvinorin B*

After identifying EOM salvinorin B as having the most bias toward G protein signaling (Figure 1), we tested it in mouse preclinical assays of anti-nociception, analgesia, and potential confounding side effects. We confirmed the prior literature (Ewald et al., 2017) that EOM salvinorin B has a spinal anti-nociception effect (tail immersion efficacy) at 1 mg/kg (paired t-test, $p<0.001$, $t=4.487$, $df=11$; Figure 2A). However, this same dose did not show supraspinal analgesia (hot plate efficacy) or any statistically significant additive effect with 5 mg/kg morphine (one-way ANOVA: $F(2,31) = 10.33$, $p=0.0004$; Dunnett's *post hoc* test, $p>0.05$; Figure 2B). This dose of EOM salvinorin B was observed to be aversive on its own (two-way ANOVA: drug effect $F(1,16)=8.578$, $p=0.0098$; time effect, $F(1,16)=11.57$, $p=0.0037$; interaction effect, $F(1,16)=3.832$,

p=0.0680; subject effect, F(16,16)=1.015, p=0.4886; Sidak's *post hoc* test, p=0.0031; Fig. 2C) and was also seen to reduce conditioned place preference when co-administered with 5 mg/kg morphine (two-way ANOVA: drug effect, F(1,10)=3.299, p=0.0994; time effect, F(1,10)=41.00, p<0.0001; interaction effect, F(1,10)=14.43, p=0.0035; subject effect, F(10,10)=3.922, p=0.0209; Sidak's *post hoc* test, p=0.0066; Fig. 2D). This same dose of EOM salvinorin B was also found to reduce both novelty-induced locomotion (unpaired t-test, p<0.01, t=4.128, df=13; Figure 2E) and morphine-induced hyperlocomotion (unpaired t-test, p<0.01, t=4.031, df= 14; Figure 2F).

Rotarod testing also revealed that this dose of EOM salvinorin B suppressed mouse motoric stability/balance after 10 minutes of administration (Figure 3). Data were analyzed by two-way ANOVA: time effect, F(1.799, 62.95)=5.478, p=0.0082; drug effect, F(3,35)=5.283, p=0.0041; interaction effect, F(6,70)=3.096, p=0.0095; subject effect, F(35,70)=1.945, p=0.0092. Dunnett's *post hoc* analysis revealed significant inhibition of motor coordination with administration of either 0.015 mg/kg nalfurafine, 5 mg/kg U50,488 or 1 mg/kg EOM salvinorin B at 10 min post injection only (p<0.01, p<0.01, and p<0.0001, respectively). Especially given this latter finding of acute inhibition of motor coordination by EOM salvinorin B, we moved to testing the second-most G protein-biased drug, nalfurafine. Furthermore, nalfurafine has been used successfully for nearly a decade in Japanese patients for the treatment of uremic pruritus (Ueno et al., 2013; Inui, 2015), and therefore (at the present time) has greater translational potential as a viable pharmaceutical adjuvant than EOM salvinorin B.

*Spinal anti-nociception by nalfurafine*

We next assessed the efficacy of the G protein-biased KOR agonist nalfurafine in the mouse warm water tail immersion test (relative to the unbiased KOR agonist U50,488) to produce

spinal anti-nociception, an outcome of KOR agonism known to be G protein-dependent (Hernandez et al., 1995; Berg et al., 2011; White et al., 2015; Abraham et al., 2018). Tail withdrawal latencies for U50,488 and nalfurafine were analyzed by one-way ANOVA: $F(6,152)=29.97$, $p<0.0001$. All three doses of nalfurafine tested produced significant increases in withdrawal latency compared with vehicle (Figure 4A; $p<0.0001$ for each; Dunnett's *post hoc* test). Administration of 1.25 mg/kg and 5 mg/kg U50,488 also increased tail withdrawal latency compared with vehicle (Figure 4A; $p<0.001$ and $p<0.0001$ respectively); while no significant difference was observed between 2.5 mg/kg U50,488 and vehicle (Holm-Sidak *post hoc* test, $p>0.05$), 5 mg/kg U50,488 elicited significantly more analgesia than 2.5 mg/kg U50,488 (Holm-Sidak *post hoc* test, $p < 0.05$). No significant difference was observed between 1.25 mg/kg and 5 mg/kg doses of U50,488 (Holm-Sidak *post hoc* test, $p>0.05$). Both 0.015 mg/kg and 0.03 mg/kg doses of nalfurafine elicited comparable anti-nociception to 5 mg/kg U50,488 (Holm-Sidak *post hoc* test, $p = 0.61$ for both).

In addition to single administration of each KOR agonist (Figure 4A), co-administration with morphine was performed to assess anti-nociceptive interactions at the spinal level (Figure 4B). Warm water tail withdrawal latencies were analyzed by one-way ANOVA: $F(3,124) = 27.36$, $p < 0.0001$. Morphine alone at 5 mg/kg produced increased latency compared with vehicle that was augmented with co-administration of either U50,488 (5 mg/kg) or nalfurafine (0.015 mg/kg) (Dunnett *post hoc* test, $p < 0.01$, $p < 0.01$, and $p < 0.05$ respectively; Figure 4B). Resultant data are also displayed as percentage of maximal possible effect (%MPE) in Supplemental Figure S1.

*Enhancement of morphine-induced supraspinal analgesia by nalfurafine co-administration*

To probe the effect of KOR agonism by nalfurafine on nociceptive response, we assessed nalfurafine co-administration on morphine-induced supraspinal analgesia *via* the hot plate test. First, nociceptive latency with administration or co-administration of the reference standard U50,488 was measured and analyzed by one-way ANOVA: $F(6,68)=8.975$, $p<0.0001$. Only the highest dose of U50,488 (5 mg/kg) produced significant augmentation of nociceptive latency compared with that produced by 5 mg/kg morphine alone (Dunnett's *post hoc* test, $p <0.001$; Figure 5A).

Nociceptive latency with co-administration of nalfurafine and morphine was also analyzed by one-way ANOVA: $F(6,86) = 30.19$, $p < 0.0001$. Co-administration of all three doses of nalfurafine tested with 5 mg/kg morphine significantly increased analgesia compared with 5 mg/kg morphine alone (Dunnett's *post hoc* test, $p < 0.0001$; Figure 5B). Furthermore, co-administration of 0.015 mg/kg nalfurafine with 2.5 mg/kg morphine produced nociceptive latencies equivalent to those produced by 5 mg/kg morphine alone (one-way ANOVA: $F(3,41) = 4.479$; $p = 0.0083$; Dunnett's *post hoc* test, $p > 0.05$; Figure 5B inset).

*Locomotor effects upon nalfurafine administration and co-administration with morphine*

Open-field locomotion represents a behavioral outcome of mesolimbic dopamine release and is increased by movement of mice into a novel environment, as well as by administration of many drugs of abuse to mice (Bardo et al., 1990; Sellings and Clarke, 2003; Bromberg-Martin et al., 2010; Fields and Margolis, 2015). Traditional KOR agonists suppress both spontaneous as well as opioid analgesic-induced locomotion (Narita et al., 1993; Chefer et al., 2005; Bruijnzeel, 2009). Suppression of total novelty-induced locomotion was analyzed by one-way ANOVA: $F(6,104) = 19.28$; $p < 0.0001$. We found that total spontaneous locomotion was significantly reduced upon

administration of 2.5 and 5 mg/kg U50,488 (Dunnett's *post hoc* test; $p < 0.0001$ and $p < 0.01$ respectively), as well as 0.015, 0.03 and 0.06 mg/kg nalfurafine (Dunnett's *post hoc* test; $p < 0.001$ and $p < 0.0001$, respectively) compared with vehicle (Figure 6A inset). No significant difference in locomotor suppression was seen between 5 mg/kg U50,488 and 0.015 or 0.03 mg/kg nalfurafine (Tukey's *post hoc* test, $p = 0.93$ and $p = 0.94$, respectively).

Modulation of total morphine-stimulated locomotion by U50,488 was analyzed by one-way ANOVA: $F(3,32) = 6.142$, $p = 0.002$. Modulation by nalfurafine was also analyzed by one-way ANOVA: $F(3,27) = 30.11$, $p < 0.0001$. Suppression of locomotion was seen with co-administration of 5 mg/kg morphine with 5 mg/kg U50,488 (Dunnett's *post hoc* test, $p < 0.01$; Figure 6D inset), as well as with all doses of nalfurafine (Dunnett's *post hoc* test, $p < 0.0001$; Figure 6E inset).

*Dose-dependent conditioned place aversion by nalfurafine*

To help assess the level of anti-therapeutic liability presented by the G protein-biased KOR agonist nalfurafine, we also measured its ability to produce conditioned place aversion in mice (Figure 7), a behavior linked to β-arrestin signaling downstream of KOR activation (Bruchas et al., 2007; Ehrich et al., 2015) and a therapeutically limiting effect of traditional KOR agonists like U50,488 (Jasinski and Mansky, 1972; Preston and Bigelow, 1993; Siebert, 1994; Greenwald and Stitzer, 1998). U50,488-induced CPA was analyzed by two-way ANOVA: test day effect, $F(1,39) = 20.41$, $p < 0.0001$; treatment effect, $F(3,39) = 8.940$, $p = 0.0001$; interaction effect, $F(3,39) = 4.648$, $p = 0.0072$; subject effect, $F(39,39) = 0.8332$, $p = 0.7143$. Nalfurafine-induced CPA was also analyzed by two-way ANOVA: test day effect, $F(1,56) = 13.83$, $p = 0.0005$; treatment effect, $F(3,56) = 1.058$, $p = 0.3741$; interaction effect, $F(3,56) = 2.443$, $p = 0.0736$; subjects effect,

$F(56,56) = 1.097$, $p = 0.3651$. We found that all doses of U50,488 produced aversion compared with vehicle (Dunnett's *post hoc* test, 1.25 mg/kg: $p < 0.01$, 2.5 mg/kg: $p < 0.0001$, and 5 mg/kg: $p < 0.001$ Figure 7A) while only the 0.03 mg/kg dose of nalfurafine produced statistically significant aversion compared with vehicle (Dunnett's *post hoc* test, $p < 0.01$; Figure 7B).

*Modulation of morphine-induced conditioned place preference by nalfurafine*

As co-administration of unbiased KOR agonists has been observed to reduce the rewarding properties of various drugs of abuse, including morphine (Kuzmin et al., 1997; Tao et al., 2006), we evaluated the ability of nalfurafine co-administration in mice to reduce the conditioned place preference produced by morphine. U50,488 results were analyzed by two-way ANOVA: test day effect, $F(1,80) = 231.0$, $p < 0.0001$; treatment effect, $F(3,80) = 0.9979$, $p= 0.3983$; interaction effect, $F(3,80) = 3.547$, $p = 0.0181$; subject effect, $F(80,80) = 1.041$, $p = 0.4286$. Nalfurafine results were also analyzed by two-way ANOVA: test day effect, $F(1,85) = 169$, $p < 0.0001$; treatment effect, $F(3,85) = 5.049$, $p = 0.0029$; interaction effect, $F(3,85) = 1.209$, $p = 0.3116$; subjects effect, $F(85,85) = 0.9096$, $p = 0.6683$. All co-administrations with 5 mg/kg morphine displayed preference for the drug-paired chamber compared with preconditioning ($p < 0.0001$ for all groups for U50,488 and nalfurafine; Figure 8).

Morphine alone (5 mg/kg) produced place preference compared with preconditioning (Sidak *post hoc test*, $p < 0.0001$; Figure 8A&B). U50,488 co-administration (at 1.25 mg/kg and 5 mg/kg) reduced preference for morphine (Dunnett *post hoc* test, $p < 0.05$; Figure 8A), although there was no significant difference between the three U50,488 doses tested (Holm-Sidak *post hoc* test, $p > 0.05$). Nalfurafine co-administration showed no effect on morphine-induced preference at 0.03 mg/kg, but produced a significant reduction in morphine preference at both the 0.015 mg/kg and 0.06 mg/kg doses (Dunnett *post hoc* test, $p < 0.05$ and $p < 0.01$, respectively; Figure 8B).

**Discussion**

Our results support nalfurafine as a G protein-biased KOR agonist that can beneficially modulate both the analgesic and rewarding properties of morphine *in vivo* upon co-administration; however, nalfurafine was also observed to produce aversion and locomotor suppression in mice. EOM salvinorin B, a KOR agonist showing greater G protein bias than nalfurafine, failed to augment morphine induced analgesia to the same degree as nalfurafine, but shared the ability to produce aversion and reduce morphine-induced CPP. The clinical viability of KOR agonists has been hampered by dysphoric effects in patients (Jasinski and Mansky, 1972; Preston and Bigelow, 1993; Siebert, 1994; Greenwald and Stitzer, 1998) -- effects linked to signaling downstream of β-arrestin mobilization (Bruchas et al., 2007; Ehrich et al., 2015). As a result, it has been hypothesized that G protein-biased KOR agonists may lack dysphoric properties (Brust et al., 2016; Liu et al., 2018), restoring their clinical viability. Our data demonstrate that, while nalfurafine and EOM salvinorin B retain the ability to produce conditioned place aversion, nalfurafine's augmentation of the analgesic effect of morphine persists at lower doses, providing evidence that G protein-biased KOR agonists may have increased clinical utility as dose-sparing agents compared with unbiased KOR agonists. Proper dose combinations may provide augmented analgesia while avoiding dysphoria and minimizing sedation.

Initial *in vitro* screening identified both nalfurafine and EOM salvinorin B as potent G-protein biased KOR agonists. Having the greatest bias factor, our initial experiments focused evaluation of EOM salvinorin B. Dosing at 1 mg/kg EOM salvinorin B, however, did not significantly augment morphine-induced analgesia in the hot plate assay. In addition, EOM salvinorin B produced CPA, reduced morphine-induced CPP when co-administered, and suppressed both novelty and morphine-stimulated locomotion. Of note is the rapid onset of suppressive action seen

in the assay of novelty-induced locomotion upon EOM salvinorin B administration. Significant reductions were present within the first 5 min post-injection of 1 mg/kg EOM salvinorin B that returned to baseline approximately 30 min later (Figure 2E). In contrast, no dose of U50,488 or nalfurafine significantly suppressed locomotion within the first 5 min compared with vehicle administration (Figure 6A). Although analgesic effects were seen at 30 min post-injection of EOM salvinorin B in our present study, as well as by others (Ewald et al., 2017), the lack of analgesic augmentation seen in the hot plate assay upon co-administration of EOM salvinorin B and morphine may be due to kinetic factors (*i.e.*, shorter half-life of EOM salvinorin B *vs* nalfurafine). Interrogation of these earlier timepoints, however, is confounded by the significant inhibition of locomotor coordination observed 10 min post-injection of EOM salvinorin B in the rotarod assay (Figure 3), potentially complicating the interpretation of hot plate results using a shorter post-injection interval. For these reasons, we chose to focus our efforts on nalfurafine. Whereas EOM salvinorin B has only been used preclinically, nalfurafine has been used safely in Japan since 2009 (Inui, 2015), giving it greater translational potential.

As previous studies have demonstrated that KOR agonist-induced increases in spinal anti-nociception are G protein-signaling dependent (Hernandez et al., 1995; Berg et al., 2011; White et al., 2015; Abraham et al., 2018), our use of the warm water tail immersion assay allowed for comparison of *in vivo* G protein-signaling between nalfurafine and the reference standard U50,488 at selected doses. A dose of 5 mg/kg U50,488 produced anti-nociception in this assay that was statistically equivalent to both 0.015 and 0.03 mg/kg doses of nalfurafine. This finding suggests that KOR-mediated G protein signaling is equivalent *in vivo* between the higher dose of U50,488 and the lowest tested doses of nalfurafine. Furthermore, we found that co-administration of 5 mg/kg U50,488 or 0.015 mg/kg nalfurafine with 5 mg/kg morphine produced augmented anti-

nociception that was equivalent between the two KOR agonists, supporting the idea of their equi-efficacious spinal-level G protein signaling at these two doses.

As the supraspinal analgesia produced by MOR-targeting analgesics represents a major factor in their clinical success (Kanjhan, 1995; Jensen, 1997), we also assessed the effect of co-administration in a non-reflexive assay of nociception (the hot plate assay). While no significant analgesic effect of U50,488 or nalfurafine alone was observed in the hot plate assay compared with morphine alone, there was a significant effect of nalfurafine upon co-administration with morphine. In contrast, only the highest dose of U50,488 (5 mg/kg) produced an augmentation of morphine-induced analgesia. To assess the dose-sparing potential of a morphine-plus-nalfurafine combination, we reduced the dose of morphine while co-administering 0.015 mg/kg nalfurafine. Co-administration of 2.5 mg/kg morphine with 0.015 mg/kg nalfurafine produced analgesia equivalent to 5 mg/kg morphine alone, suggesting that a combination of morphine and nalfurafine may reduce the dose of morphine required to achieve similar analgesia by half.

A potential explanation of the observed analgesic effects of nalfurafine is that the locomotor-suppressing property of nalfurafine might hinder expression of nociceptive behaviors (*e.g.*, jumping or paw flicking/licking), thereby falsely increasing nociceptive latency measurements. However, in our measurements of morphine-stimulated hyperlocomotion, 5 mg/kg U50,488 was observed to suppress locomotion to a similar extent as both 0.015 and 0.03 mg/kg doses of nalfurafine (Figure 6), yet nalfurafine produced greater augmentation of analgesia (Figure 5). In addition, rotarod assays revealed decreased locomotor coordination upon co-administration of morphine with either U50,488 or nalfurafine at 10 min post-injection only; these locomotor coordination effects were not present at 30 min post injection: namely, the timepoint at which hot plate assessment of nociceptive behaviors was conducted. As morphine-stimulated

hyperlocomotion in mice is strongly linked with mesolimbic dopamine release (Bromberg-Martin et al., 2010; Fields and Margolis, 2015), the effects seen in this locomotion assay upon co-administration likely reflect a reduction in the motivating effects of morphine. In contrast, the rotarod assay provides a direct stimulus for locomotion (not falling from the rotating rod) and thereby more directly evaluates locomotor coordination, suggesting that the ability of these mice to locomote at 30 min post injection is not significantly disrupted and making a locomotor confound to nociception less likely. In this light, future studies are planned to investigate the effects of nalfurafine and MOR agonist co-administration on pain-suppressed behaviors to further investigate this potential confound and better characterize the analgesic interactions of nalfurafine with MOR-targeting drugs.

As 5 mg/kg U50,488 or 0.015 mg/kg nalfurafine each produced equivalent analgesic augmentation when co-administered with 5 mg/kg morphine in the spinal anti-nociception (tail withdrawal) assay, the increased ability of nalfurafine to augment morphine-induced analgesia in the supraspinal (hot plate) assay may indicate a beneficial supraspinal interaction between morphine and nalfurafine. Early work with site-specific administration of MOR and KOR agonists demonstrated analgesic synergism when given intrathecally (*it*) but not intracerebroventricularly (*icv*) (Ren et al., 1985; Sutters et al., 1990; Miaskowski et al., 1992; Miaskowski et al., 1993). Importantly, these studies utilized unbiased KOR agonists such as dynorphin and U50,488. These findings suggest that the beneficial analgesic interaction seen with administration of nalfurafine and morphine may stem from a reduced engagement of β–arrestin at supraspinal sites, rather than a difference in G protein-signaling *per se*.

To assess the potential clinical viability of this co-administration paradigm, we evaluated doses of nalfurafine and U50,488 for sedation and dysphoria. In the present study, statistically significant

reductions in spontaneous locomotion were seen with all KOR agonists tested except 1.25 mg/kg U50,488. This observation of sedation persisted when these KOR agonists were co-administered with morphine. However, post-marketing surveillance of nalfurafine reports a low incidence of somnolence (1%) at doses safe and efficacious for anti-pruritus in human patients (Kozono et al., 2018; Siderovski and Setola, 2018), suggesting that sedation may not necessarily prove to be an anti-therapeutic effect of nalfurafine co-administration with a MOR-targeting analgesic.

Aversion to KOR agonists in the conditioned place aversion assay (a rodent-based metric of dysphoria) has been linked to signaling downstream of β-arrestin (Bruchas et al., 2007; Ehrich et al., 2015), and previous work has demonstrated a lack of CPA to nalfurafine (Tsuji et al., 2001; Liu et al., 2018). In our CPA assays with nalfurafine, only 0.03 mg/kg produced significant aversion, as compared with U50,488 which produced significant aversion at all doses tested. Observed decreases in aversion with high doses of KOR agonist has been demonstrated in a previous study (Robles et al., 2014) and could reflect the appearance of cognitive deficits dependent on the high dosing (Castellano et al., 1988; Daumas et al., 2007; Carey et al., 2009; Paris et al., 2011). Of note is our finding that 0.03 mg/kg nalfurafine produced significant conditioned place aversion. This result is congruent with recent work demonstrating that the G protein biased KOR agonist RB-64 produces aversion in both wild-type and β-arrestin-2 knockout mice (in addition to the unbiased KOR agonists U69,593 and salvinorin A (White et al., 2015)), suggesting that aversion to KOR agonists may be produced in part through mechanisms independent of β-arrestin-dependent signaling. G protein-biased KOR agonists might thus retain the ability to produce aversion, although less potently than unbiased agonists.

KOR agonists are known to reduce the rewarding properties of a variety of drugs of abuse (Kuzmin et al., 1997; Lindholm et al., 2001; Tsuji et al., 2001; Tao et al., 2006; Morani et al.,

2009b; Ewald et al., 2017; Townsend et al., 2017b), presenting a second possible benefit of combination KOR and MOR agonist therapy. Using the CPP assay, we found that nalfurafine reduces the rewarding properties of 5 mg/kg morphine at 0.015 and 0.06 mg/kg, while 0.03 mg/kg produced no effect on morphine-induced place preference. Although a dose of 0.03 mg/kg was reported to reduce morphine CPP in a previous study (Tsuji et al., 2001), a potential source of this difference could be our use of inbred C57BL/6J mice, as opposed to outbred ddY male mice used by Tsuji and colleagues. Indeed, strain differences in response to KOR agonists, as well as to psychostimulant drugs of abuse, have previously been documented (*e.g.*, (Castellano et al., 1988; Mouri et al., 2012)).

Administration of U50,488 displayed a similar pattern in CPP assays, with doses of 1.25 and 5 mg/kg U50,488 suppressing morphine-induced place preference, while 2.5 mg/kg U50,488 did not result in a statistically significant suppression of CPP. Similar to results seen in CPA, the reduction in morphine-induced CPP at the highest doses of each KOR agonist may be explained in part by cognitive disruption. Paris and colleagues have previously shown that U50,488, at a dose that reduces CPP for cocaine, produces deficits in normal object recognition (Paris et al., 2011). In addition, U50,488H injected bilaterally into the CA3 region of the hippocampus is reported to attenuate context-induced fear conditioning as well as the ability to identify a new platform location in the water maze task (Daumas et al., 2007). The results from these prior studies suggest that a reduced ability to pair stimulus with context may be responsible for the decreased conditioned place preference for morphine seen with the highest doses of KOR agonists used in our study.

Effects seen with the low and middle dose of each KOR agonist tested were opposite of those expected. Interestingly, the doses of both drugs producing the greatest level of aversion produced the least suppression of morphine-induced CPP. While extensive prior work has focused on KOR agonist effects on cocaine CPP (Suzuki et al., 1992; Redila and Chavkin, 2008; Morani et al., 2009a), there is a paucity of published work investigating their effects on opioid analgesic-induced CPP, with few (if any) studies using multiple doses of a KOR agonist. Outside of the work by Tsuji and colleagues referenced above (Tsuji et al., 2001), another group demonstrated that 1 mg/kg U50,488 reduced CPP for 5 mg/kg morphine, although other doses of U50,488 were not assessed (Masahiko et al., 1993). A drawback of the CPP procedure is its relatively low sensitivity to dose magnitude (Napier et al., 2013), making dose-response relationships difficult to properly establish; however, the effects reported here indicate an interaction between the dose of KOR agonist and the rewarding properties of morphine. As pretreatment with U50,488 has been shown to increase CPP for cocaine (McLaughlin et al., 2006; Ehrich et al., 2014), it is possible that differences in the timing of KOR agonist effects at different doses in relation to the rewarding effects of morphine play a role in the observed U-shaped dose-response pattern. A more detailed interrogation of the effects seen in CPP with an expanded dose range of nalfurafine and varied pretreatment intervals could aid in determining the pharmacodynamic and pharmacokinetic factors involved.

Taken together, our data suggest that nalfurafine may be an effective dose-sparing adjuvant for traditional MOR-targeting analgesics. As all doses of nalfurafine tested provided similar augmentation of morphine's analgesic effect, lower doses than implemented here may provide adequate analgesic synergism while avoiding dysphoria and sedation. Nalfurafine is used in the treatment of uremic pruritus at doses that produce low rates of sedation, insomnia, constipation,

psychosis and other common KOR related side effects (Kozono et al., 2018; Siderovski and Setola, 2018). The anti-pruritic dose in mice extends as low as 5 μg/kg (Liu et al., 2018). As a dose of 0.015 mg/kg effectively reduces morphine CPP without significant aversion, it is possible that doses already used in human subjects to treat pruritus may produce the benefits of co-administration described herein while avoiding significant anti-therapeutic effects. Although the signaling pathways involved in both the intended and unintended effects of KOR agonism require further investigation, the reduced side-effect profile of nalfurafine as compared with unbiased KOR agonists suggests that G protein-biased KOR agonists may become clinically relevant adjuvants for opioid-based pain therapies, particularly for acute pain. Additional studies are planned to investigate the effects of chronic co-administration on analgesic tolerance-, withdrawal-, and reward-related behaviors.

**Acknowledgements**

We thank Dr. Gilad Barnea (Brown University) for furnishing HTLA cells for Tango assays, and Dr. Liz Engler-Chiurazzi for access to, and advice with, the WVU Rodent Behavioral Core facility.

**Authorship Contributions**

*Participated in research design*: Kaski, White, Gross, Trexler, Kinsey, Siderovski, and Setola

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*Wrote or contributed to the writing of the manuscript*: Kaski, White, Gross, Kinsey, Kenakin, Siderovski, and Setola

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**FOOTNOTES:**

***Financial support**

This work was supported, in part, by the E.J. Van Liere Medicine Professor Endowment (to D.P.S.) and by research grants from the National Institute on Drug Abuse to T.E.P. [R01 DA018151], to D.P.S. [R01 DA048153], and to S.G.K [R03 DA039335]. J.D.G. and S.W.K. each acknowledge prior support from an National Institute of General Medical Sciences Behavioral & Biomedical Sciences training grant [T32 GM081741] and current support from National Institute on Drug Abuse predoctoral NRSA fellowships [F31 DA043331 and F30 DA044711, respectively].

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**Legends for Figures:**

**Figure 1**. **Nalfurafine is a potent G protein biased KOR agonist.** Comparison of G protein and β-arrestin signaling outcomes with a variety of KOR ligands (at indicated concentrations) as compared with the reference standard U50,488. **(A)** G protein signaling was assessed with a GloSensor assay of cyclic AMP inhibition. **(B)** β-arrestin recruitment to the activated KOR was assessed via the Tango assay. **(C,D)** Results from GloSensor and Tango assays used to calculate bias factors for each compound from observed maximal efficacy ($E_{max}$ or "Max") and potency ($EC_{50}$) values. $\Delta\Delta\log(Max/EC_{50})$ values, with their 95% confidence intervals (CIs), are plotted in panel D for each compound to indicate relative (to U50,488) bias towards G protein signaling. All compounds were tested in triplicate alongside the reference standard U50,488. $N \geq 3$ for all compounds.

**Figure 2. Evaluation of EOM salvinorin B as a potential dose-sparing adjuvant for morphine.** **(A)** EOM salvinorin B (1 mg/kg) produced spinal anti-nociception as measured by warm water tail withdrawal latencies significantly increased over vehicle control, (n = 12). Data were analyzed by paired t-test, ***, $p < 0.001$. **(B)** EOM salvinorin B did not significantly augment the analgesic effect of morphine, n = 8-15 for all groups. Data were analyzed by one-way ANOVA. **(C, D)** EOM salvinorin B produced significant conditioned place aversion (panel C) and significantly reduced morphine-induced conditioned place preference (panel D) (n=6-11 for all groups). Data were analyzed by two-way ANOVA, **, $p < 0.01$. **(E, F)** EOM salvinorin B suppressed both novelty-induced (panel E) and morphine-stimulated locomotion (panel F) (n = 7-8 for all groups). Data were analyzed by unpaired t-test, **, $p < 0.01$.

**Figure 3. Rotarod assay of motoric effects of tested KOR agonists as adjuvants to morphine.** The rotating rod was set to begin rotating at 4 rpm and accelerate to 40 rpm over the course of 5 min. Mice were trained on the rotarod on day 1 in two 5-min sessions. On day 2, the baseline latency to fall off the rod was determined and then mice were injected with either vehicle or drug as indicated in the inset legend. Mice were then assessed at 10, 30 and 60 min post injection. Data are reported as percent of baseline performance. Adding U50,488 (5 mg/kg), nalfurafine (0.015 mg/kg) or EOM salvinorin B (1 mg/kg) to 5 mg/kg morphine each produced a decrease in the time on the rod 10 min after co-administration, but not at 30 or 60 min post injection. n = 8-11 for all groups. Data were analyzed by two-way ANOVA; **, $p<0.01$; ****, $p<0.0001$.

**Figure 4. Spinal nociception in C57BL/6J mice is blunted by the KOR agonists U50,488 and nalfurafine, and both KOR agonists enhance the anti-nociceptive effect of morphine. (A)** Mice were treated with vehicle 30 min prior to establishing baseline latencies, then treated with indicated doses of nalfurafine or U50,488 30 min prior to testing. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison *post hoc* test to assess for differences from the pooled vehicle. Holm-Sidak multiple comparison *post hoc* test was used to assess for differences between treatment groups (n= 10-22 for all treatment conditions; ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; ###, $p < 0.001$; ####, $p < 0.0001$.). **(B)** Mice were treated with 5 mg/kg morphine alone, or in combination with either 5 mg/kg U50,488 or 0.015 mg/kg nalfurafine. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison *post hoc* test to assess for differences from morphine alone (n=21-22 for all treatment groups). Data in Figure 4 are also represented as percent of maximal possible effect (%MPE) in Supplemental Figure S1.

**Figure 5. Nalfurafine co-administration potentiates morphine-induced supraspinal analgesia in C57BL/6J mice.** Mice were treated with indicated doses of morphine, nalfurafine, U50,488, or a combination of morphine (5 mg/kg) with either nalfurafine or U50,488. Latency to nociceptive response was recorded and compared with the response to saline [%MPE = (test response - baseline)/(30 sec - baseline); thus, 100% MPE = 30 sec with no sign of nociception]. All data sets were analyzed by one-way ANOVA with Dunnett' multiple comparison *post hoc* test to assess differences from 5 mg/kg morphine. *, $p < 0.05$; ***, $p < 0.001$; **** , $p < 0.0001$. **A.** Tests of the reference standard U50,488: n=18 for 5 mg/kg morphine; n = 7-16 for all other conditions. **B.** Tests of the G protein-biased nalfurafine: n = 27 for 5 mg/kg morphine; n = 8-18 for all other conditions. Inset shows %MPE values for administration of 0.015 mg/kg nalfurafine with 1.25, 2.5, or 5 mg/kg morphine normalized to 5 mg/kg morphine alone (n = 8-15 for all groups).

**Figure 6**. **Nalfurafine administration affects both spontaneous and morphine-induced locomotion.** Assessment of open-field locomotion as elicited by environmental novelty or morphine administration. **(A)** For assessment of spontaneous locomotion, mice were administered vehicle or indicated single drug and immediately placed into the open-field chamber for 60 min. **(B, C)** For assessment of morphine-induced locomotion, mice were again habituated to the chamber for 30 min (on day 3) before receiving 5 mg/kg morphine with vehicle, or 5 mg/kg morphine with 1.25, 2.5 or 5 mg/kg U50,488 (panel B), or 5 mg/kg morphine with 0.015, 0.03 or 0.06 mg/kg nalfurafine (panel C) and assessed for locomotor behavior for an additional 60 min. Locomotion was measured by number of IR-beam breaks per 5 min. Data are presented as percent of pooled vehicle locomotion over the 60 min post injection for suppression of novelty-induced locomotion. Total locomotion data were analyzed by one-way ANOVA with a Dunnett's *post hoc*

analysis to compare treatment groups with the vehicle alone or vehicle + morphine condition and Tukey's *post hoc* analysis to assess difference between treatment conditions for novelty locomotion. Novelty-induced locomotion n: vehicle = 41, all other conditions, n = 7-16. Morphine suppressed locomotion n = 7-11 for all conditions. ns, not significant; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

**Figure 7. Dose-dependent production of conditioned place aversion (CPA) in C57BL/6J mice by nalfurafine administration.** Mice were acclimated to the two-chambered apparatus and allowed to display a side preference. Then, mice were treated with saline (in their non-preferred chamber) or drug (in their preferred chamber) and confined to that chamber. Vehicle and drug were given on alternate days for four days. On test day, the mice were allowed free access to either chamber. The preference for the drug-paired chamber was measured as the time spent in the drug-paired chamber minus the time spent in the vehicle-paired chamber. **(A)** Mice treated with the reference standard U50,488 displayed a significantly reduced preference for the drug-paired chamber (**, $p < 0.01$; ****, $p < 0.0001$, ***, $p < 0.0004$, n=8-15 for all groups). **(B)** Mice treated with 0.03 mg/kg nalfurafine, but not 0.015 or 0.06 mg/kg nalfurafine, displayed reduced preference for the drug-paired chamber. Data were analyzed by one-way ANOVA with a Dunnett's multiple comparisons test comparing with vehicle (*) or a Sidak test to compare between doses of U50,488 or nalfurafine. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, n = 8-26 for all groups).

**Figure 8**. **Nalfurafine, like the conventional KOR agonist U50,488, reduces morphine-induced conditioned place preference (CPP) in C57BL/6J mice.** Mice were acclimated to the two-chambered apparatus and allowed to display a side preference. Then, mice were treated with vehicle (in their preferred chamber) or drug (in their non-preferred chamber) and confined to that chamber for 30 or 50 min (for U50,488 or nalfurafine, respectively). Vehicle or morphine was given on alternate days for four days. On test day, the mice were allowed free access to both chambers. The preference for the drug-paired chamber was measured as the time spent in the drug-paired chamber minus the time spent in the vehicle-paired chamber. **(A)** Mice co-administered morphine (5 mg/kg) and U50,488 (1.25 or 5 mg/kg) displayed reduced preference for the drug-paired chamber (n=18-25 for all groups). **(B)** Mice co-administered morphine (5 mg/kg) and nalfurafine (0.015 or 0.06 mg/kg) displayed reduced preference for the drug-paired chamber (n=18-25 for all groups). Data were analyzed by two-way ANOVA with a Sidak's multiple comparisons test comparing with preconditioning (*), and a Dunnett's test comparing with morphine alone (#): #, $p < 0.05$; ##, $p < 0.01$; ****, $p < 0.0001$.

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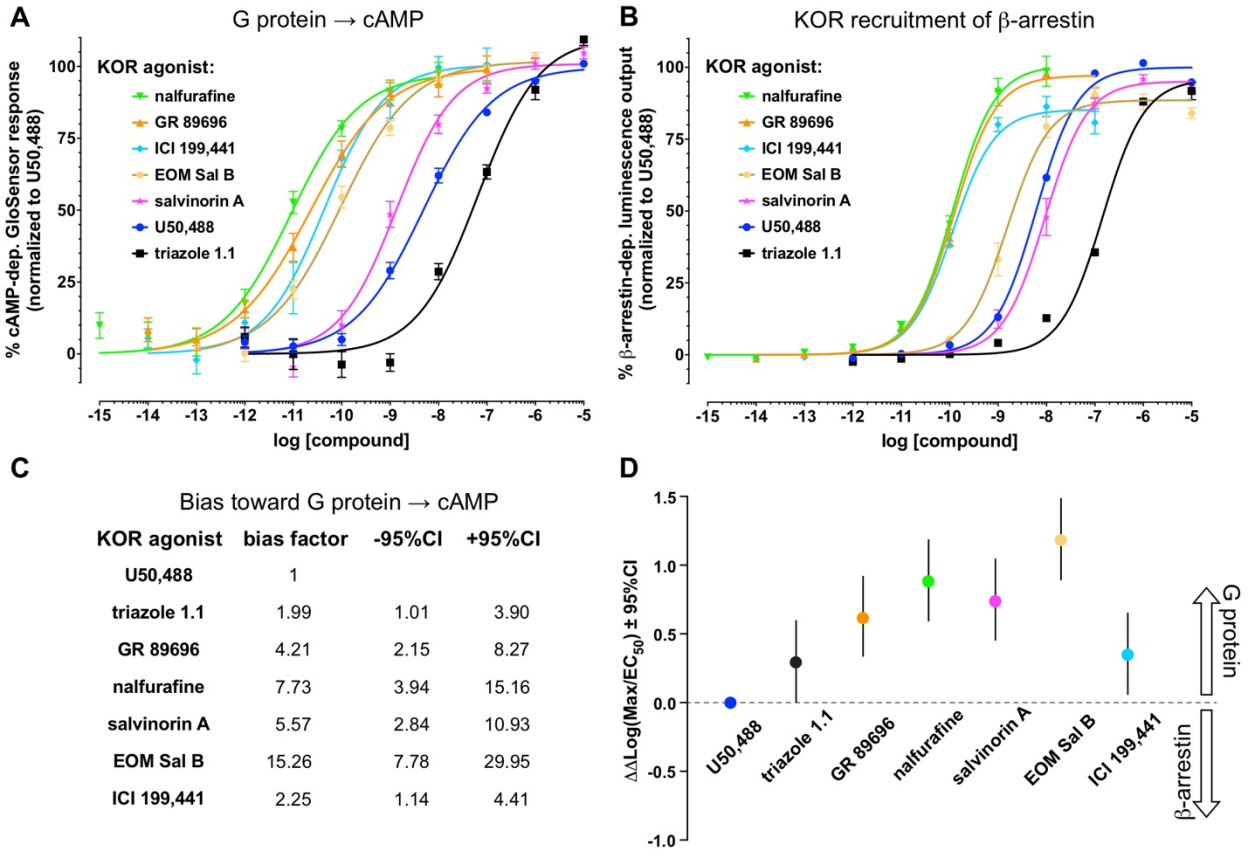


C

Bias toward G protein → cAMP

| KOR agonist | bias factor | -95%CI | +95%CI |
|---|---|---|---|
| U50,488 | 1 | | |
| triazole 1.1 | 1.99 | 1.01 | 3.90 |
| GR 89696 | 4.21 | 2.15 | 8.27 |
| nalfurafine | 7.73 | 3.94 | 15.16 |
| salvinorin A | 5.57 | 2.84 | 10.93 |
| EOM Sal B | 15.26 | 7.78 | 29.95 |
| ICI 199,441 | 2.25 | 1.14 | 4.41 |




**Figure 1**

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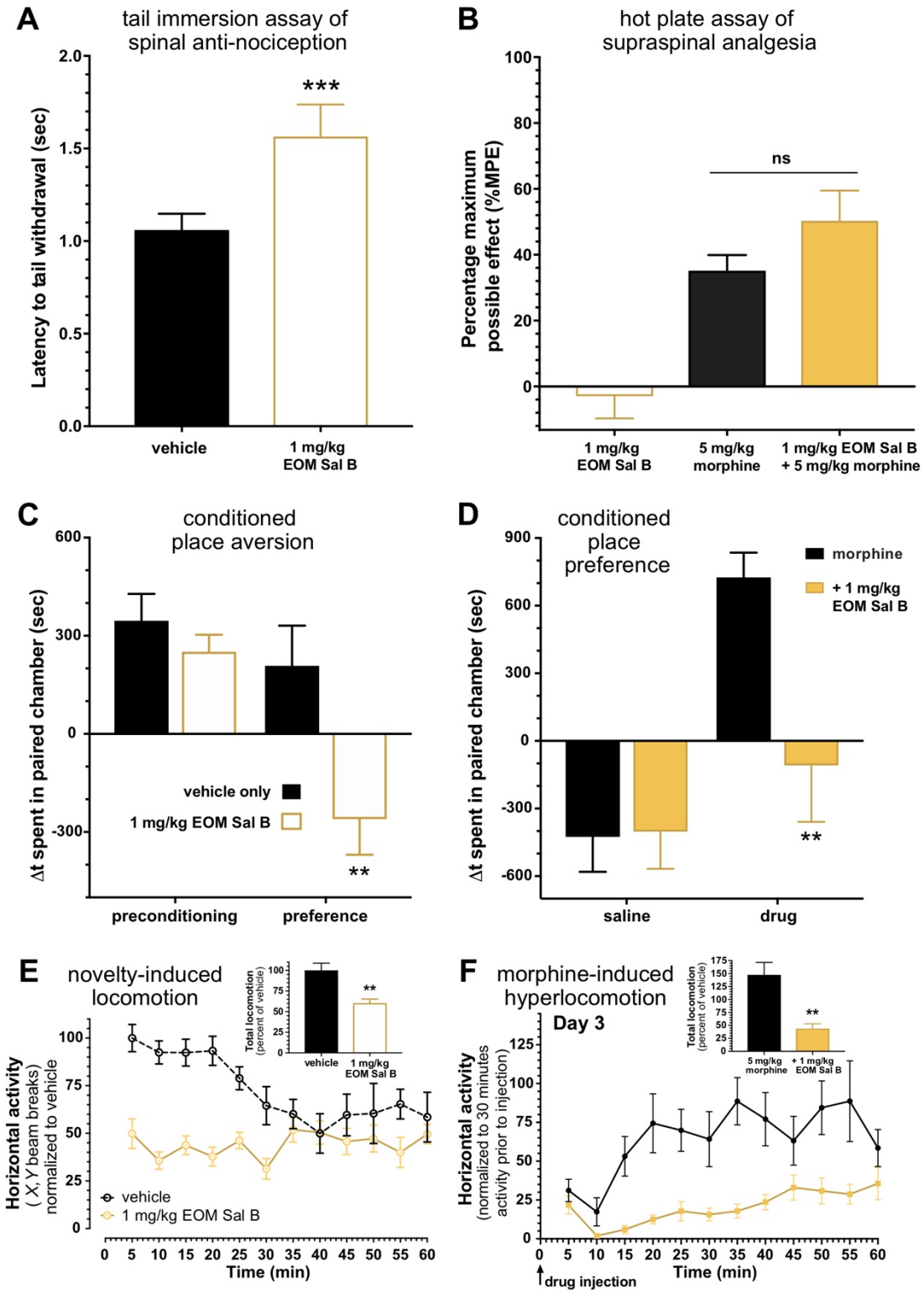


**Figure 2**

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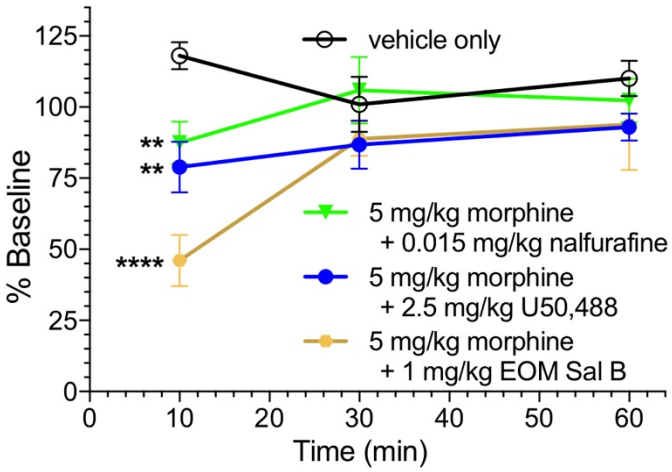


**Figure 3**

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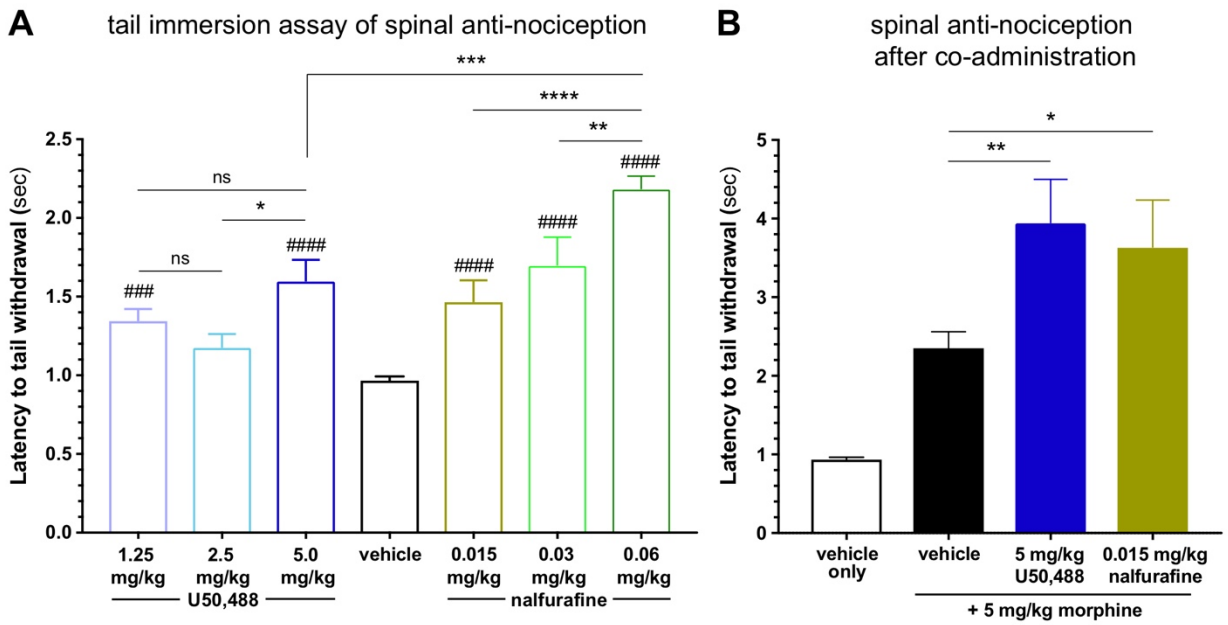


**Figure 4**

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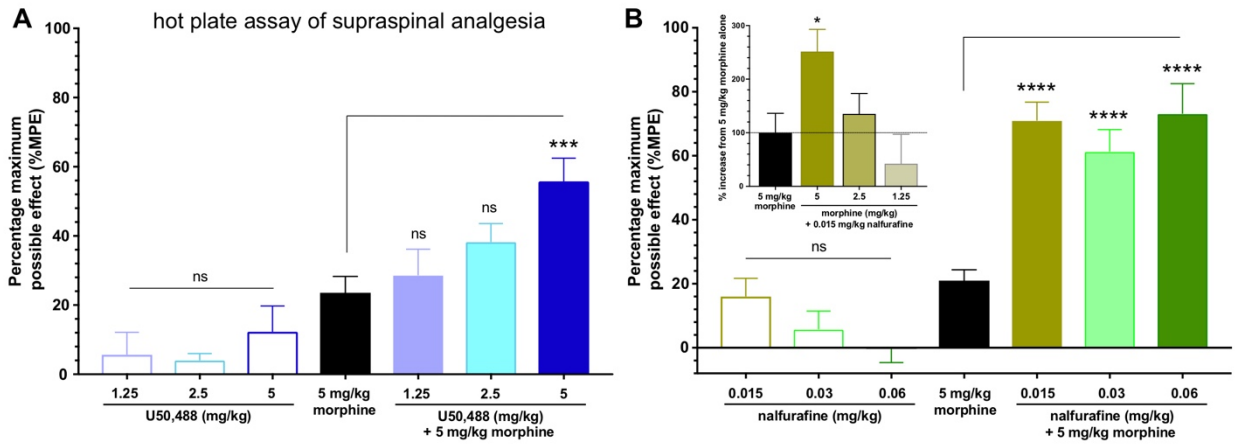


**Figure 5**

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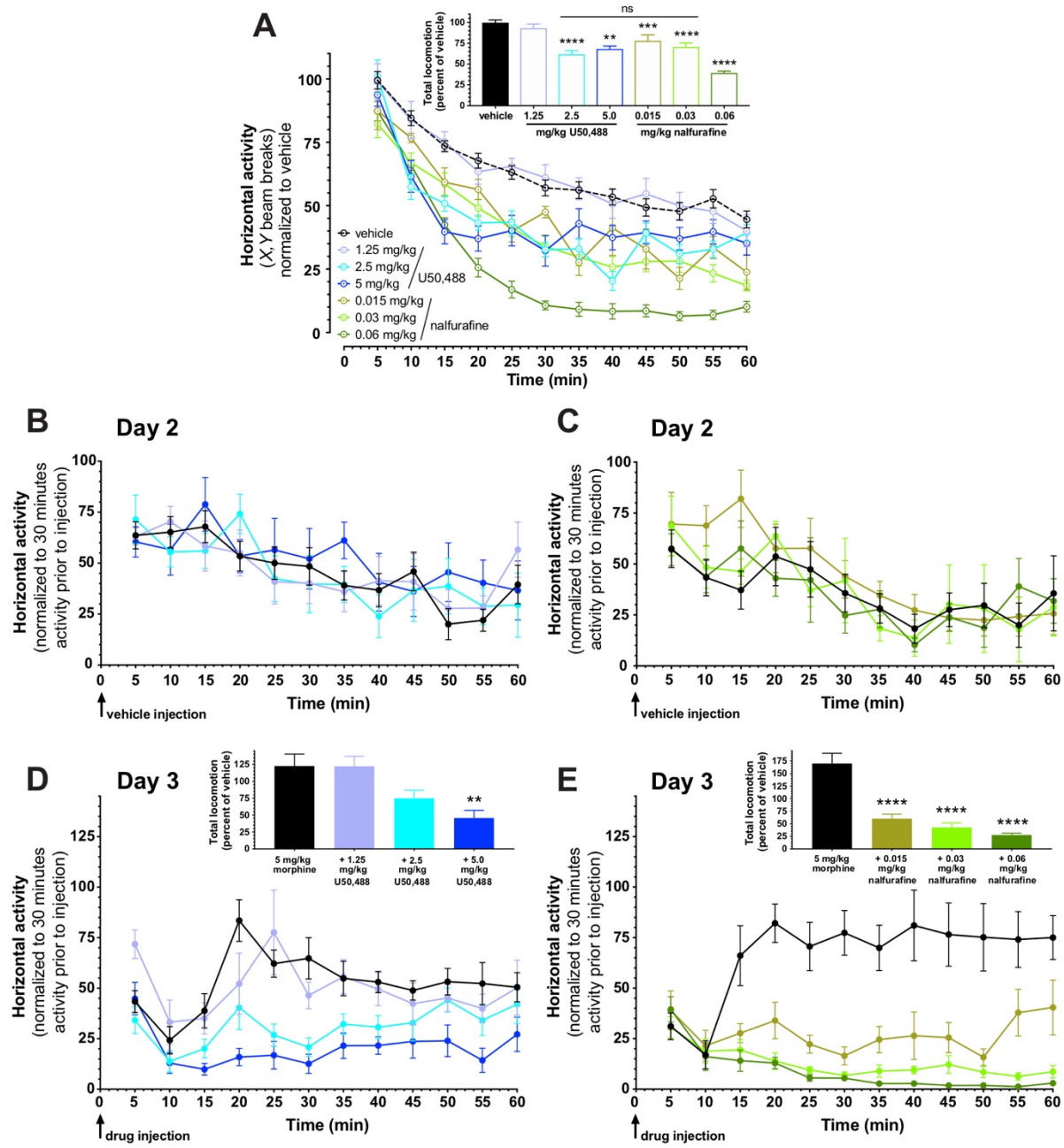


**Figure 6**

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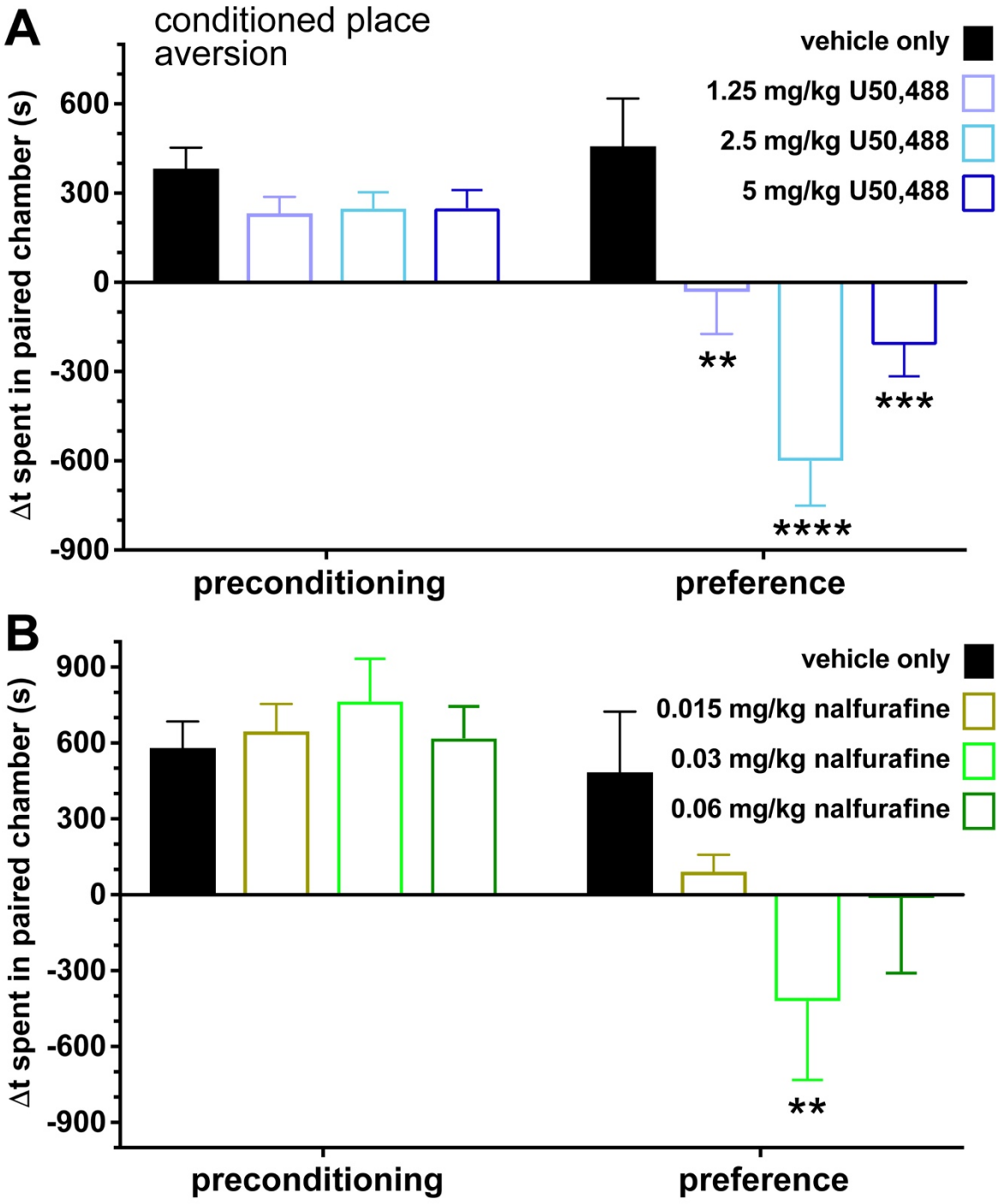


**Figure 7**

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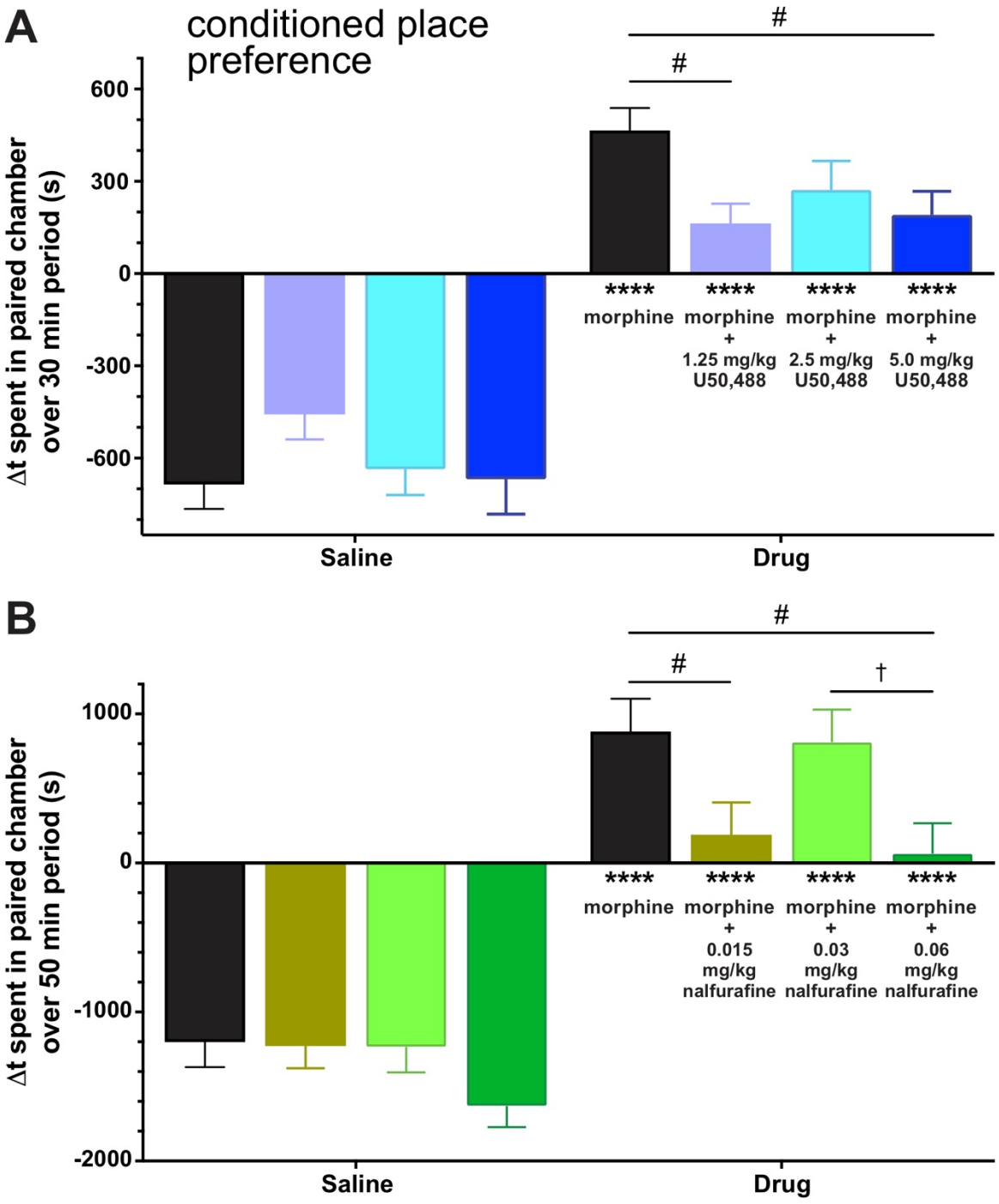


**Figure 8**

# Preclinical testing of nalfurafine as an opioid-sparing adjuvant that potentiates analgesia by the mu opioid receptor-targeting agonist morphine*

Shane W. Kaski, Allison N. White, Joshua D. Gross, Kristen R. Trexler, Kim Wix, Aubrie A. Harland, Thomas E. Prisinzano, Jeffrey Aubé, Steven G. Kinsey, Terry Kenakin, David P. Siderovski, and Vincent Setola

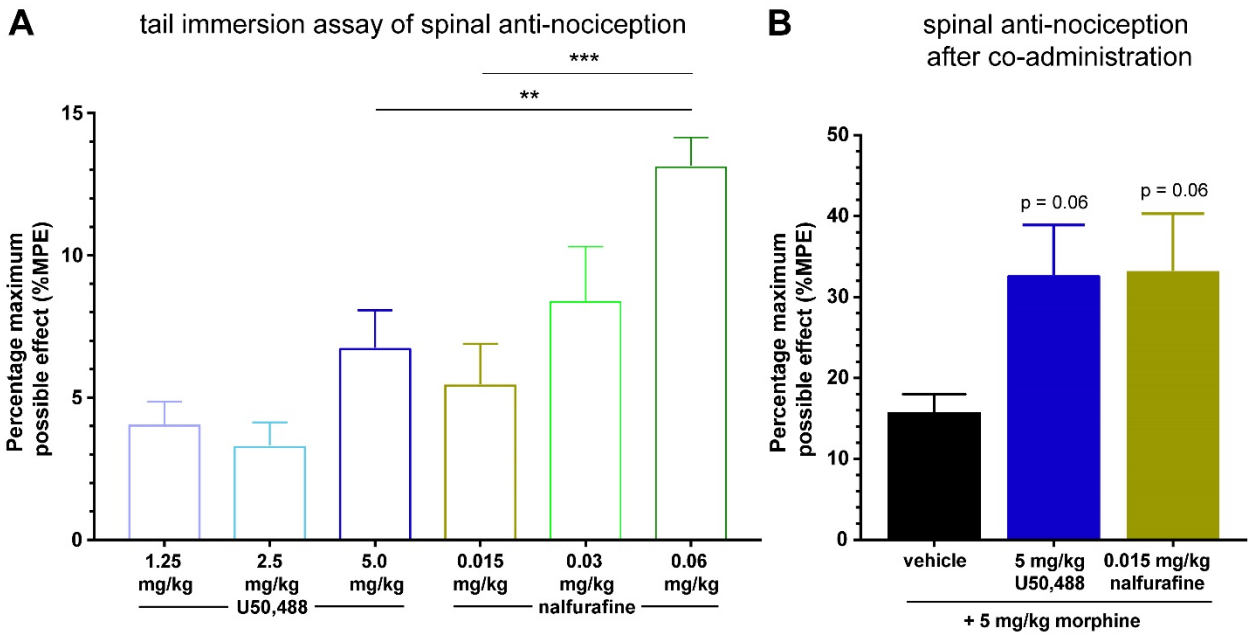


**Figure S1. Data for tail immersion assay of spinal anti-nociception presented as %MPE.** Experimental procedures and n values are identical to those of Figure 4. Data are expressed as % maximum possible effect (MPE) utilizing the following equation: %MPE = (treatment latency – baseline latency)/(10 sec – baseline latency)*100. Data were analyzed by one way ANOVA: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.