Phosphoproteomic approach for agonist-specific signaling in mouse brains: mTOR pathway mediates κ opioid aversion

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SUPPLEMENTARY INFORMATION

Supplementary Methods

Animals. Male CD-1 mice (Charles River Laboratories, Wilmington, MA), weighing between 30-35 g, were used for most of the behavioral and all the phosphoproteomics experiments. Adult male MOR knockout (Matthes *et al*, 1996) and wildtype mice in C57BL/6 background (25-30 g) were used in some experiments. Mice were housed in the animal facility under a 12 h light/dark cycle with lights on at 7:00 AM and allowed food and water *ad libitum* until the time of each experiment. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with approval from Temple University School of Medicine Institutional Animal Care and Use Committee. All experimental groups contained at least 8 mice. Each animal used was drug-naïve and used only once.

Compound 48/80 scratching test. The test was performed as we described (Wang *et al*, 2005). After acclimation to individual rectangular observation boxes for 1 h, mice were then injected s.c. in the flank with either vehicle or a KOR agonist followed by 20 min later injection (s.c.) with 0.1 ml compound 48/80 (0.5 mg/ml; 50 μg) into the nape. Starting 1 min post-injection, the number of bouts of hind leg scratching movements directed to the neck was

counted for 30 min. For each group of mice, the mean values for scratching were normalized to relative % reduction of scratching and then plotted vs. dose of KOR agonist (on a log scale) using the following equation:

$$\frac{\text{mean number of scratches by control group - mean number of scratches by treatment group}}{\text{mean number of scratches by control group}} x100$$

Formalin test. This is the most commonly used model of tonic postoperative pain in current analgesic research and the test was performed as described (Murray *et al*, 1988). Animals were treated with vehicle or varying doses of a KOR agonist (s.c.) and then placed in cylindrical glass jars (1/jar) (14 cm in diameter). Five min later, the mouse was mildly sedated with 3-5% isoflurane and injected s.c. with 20 µl of a 5% formalin into the plantar region of the right hind paw. The mice were returned to their jars and the time each animal licked/groomed the formalin-injected paw was recorded from 15 min to 35 min post-injection. Results for each mouse were normalized into % analgesia using the following equation:

$$\frac{\text{mean licking time of control group - mean licking time of treatment group}}{\text{mean licking time of control group}} x100$$

Determination of A_{50} values of analgesia and anti-scratch effects: The A_{50} values were calculated by plotting [dose of agonist] against % analgesia or anti-scratch effect and using linear regression to obtain a best-fit line (Prism 6.0, GraphPad Software, La Jolla, CA).

Conditioned place aversion (CPA). The experiment was performed with procedures adapted from our published method for conditioned place preference (Xu *et al*, 2013). During the pre-test, each animal could roam freely between the two compartments for 15 min, and the time

the animal spent on each side was recorded. In the conditioning phase, two sessions per day were conducted, with a minimum of 4 h between the morning and afternoon conditioning sessions, for a total of 12 pairings. The timing of the drug and vehicle injections was alternated. On the day of the post-test, the animals were once again allowed to freely roam between the two compartments for 15 min, and time spent on each side was recorded. The preference score was calculated by subtracting the time the animal spent during the post-test on the drug-paired (initially preferred) side minus the pre-test score. A negative preference score indicates aversion, while a positive score indicates preference. In some experiments, mice were injected with vehicle of rapamycin (5 mg/kg, s.c.) 30 min before administration of U50,488H or saline on days 1, 3, 5 during conditioning.

Intracranial self-stimulation (ICSS). ICSS experiments were performed on adult mice as described by Muschamp et al. (Muschamp *et al*, 2011). Mice were stereotactically implanted with a monopolar stimulating electrode (PlasticsOne, Roanoke, VA) located in the medial forebrain bundle (from bregma: AP:-1.9, ML: +0.8, DV: -4.8 mm). After five days of recovery, mice were trained to respond to electrical stimulation using computer-controlled operant chambers housed in ventilated, sound-attenuating cabinets (Med Associates; St. Albans, VT). Mice were trained in 115 minute daily sessions at the lowest current that would sustain responding at a rate of ~1 response/sec (± 10%; FR1 schedule of reinforcement). This preferred current was then maintained through testing in which mice were permitted 50 seconds to respond for one of fifteen stimulation frequencies (158-34 Hz) presented in descending order following the 'rate-frequency' procedure for estimating the minimum frequency at which stimulation supports operant responding (i.e. reward threshold or 'θ_0').

Reward threshold was computed for each subject using a least-square line of best fit analysis. Drug testing began when reward thresholds reached a stable performance criteria (± 10% for 3 consecutive days). For drug treatment sessions, doses of U50,488H (0, 0.25, 0.5, 1.0 or 2.5 mg/kg, s.c.) or nalfurafine (0, 5, 10, 20 or 40 μg/kg, s.c.) were administered following a Latin Square design 45 minutes after beginning the task. Thresholds were quantified from performance during 15-min test sessions. Percent baseline was computed as: (threshold after drug/threshold before drug)*100.

Locomotor activities. Activities were measured according to our published procedures (Xu *et al*, 2013) using a Home Cage Locomotor Activity System (Omnitech Electronics Inc., Columbus, OH) and eight individual activity monitors under normal laboratory lighting conditions. Mice were treated s.c. with saline, U50,488H (5 mg/kg) or nalfurafine (20 μg/kg) (2.5x A_{50} values in the anti-scratching test) and put into locomotor chambers right after injections. Total locomotor activities were continuously monitored over a 60-min period.

Rotarod test was performed according to a published procedure (White *et al*, 2015) using a mouse rotarod apparatus (Ugo-Basile, Stoelting Co., Wood Dale, IL). Adult CD1 mice were trained to run on a rotarod apparatus one day before the test day. On the training day, mice were placed on the rotarod for 5 min with the rotation revolution changing from 5 to 50 rpm. Each mouse was trained for 2-3 trials with a break of 1 min between trials. About 40-50% of mice could stay on the rotarod for more than 240 sec (80% of the 5-min period) after trials and were used next day. One the test day, these mice were tested on the rod first to measure their baselines. More than 90% of them, which had a baseline above 240 sec, were injected

subcutaneously with a KOR agonist or saline. Ten, 20, 30 and 40 min after the injection, mice were repeatedly tested for the time they stayed on the rotarod for 5 min. Results are shown as the percentage of time staying on the rod following drug or vehicle administration compared with the baseline.

Phosphoproteomic analyses of the mouse brain regions following treatment with U50,488H and nalfurafine. Adult male CD-1 mice (30-35 g) were injected (s.c.) with vehicle, U50,488H (10 mg/kg), or nalfurafine (20 μg/kg) (n=3 each) and euthanized 30 min later. Brains were removed and brains regions were dissected, frozen immediately, solubilized in 4% SDS in 50 mM Tris-HCl buffer (pH7.4) and stored at -20°C until analysis. Proteins from the brain samples were precipitated with cold acetone (v/v 80%), re-dissolved in a 2,2,2-trifluoroethanol (TFE) buffer, and transferred to equal amount of distilled water inside a 96-well plate. After protein quantification, equal amounts (~2mg) of samples were digested by trypsin and lysC overnight. Digested phosphopeptides were then enrich as described previously (Humphrey *et al*, 2015; Sharma *et al*, 2014). Briefly, TiO_2 beads were incubated with samples and washed in organic buffer for 4 times before elution in high pH. The eluted phosphopeptides were immediately dried with Speedvac and desalted. Samples were then measured using Thermo Scientific Qxactive HF orbitrap, and processed with Maxquant (version 1.5.5.2) (Cox and Mann, 2008). The default setting was used with a few exceptions. Briefly, in the “Group-specific parameters”, the digestion mode was set to Trypsin/P, and allowed maximal of 3 miss cleavage events; the modifications mode was set to include Phospho (STY). In the “Global parameters”, mouse fasta file was used as the search space. Using these setting, Maxquant extracted identity and intensity information of each phosphopeptide from mass spectra, and produce

data tables containing this information. The resulted data table was processed bioinformatically using R and Perseus (version 1.5.2.17) (Tyanova *et al*, 2016).

The annotation matrix algorithm was applied under the Perseus environment (version 1.5.0.1). Briefly, individual data set was normalized against the median of their respective control groups and was annotated with Gene ontology terms or KEGG pathways terms based on their protein identification. The annotation matrix is an expansion of 1D annotation enrichment described by Cox and Mann (Cox and Mann, 2012). Briefly, phosphosites of proteins in the one annotated category was tested against the remaining phosphosites through a Wilcoxon-Mann-Whitney U test. We then set p-value = 0.001 as the cutoff, and obtained the median differences between the significantly regulated category and the background. Since all tests performed so far are on every single replicate in different experimental condition, we performed one sample t test on each experimental condition against 0. The obtained p-value indicates if each enriched category was reproducibly regulated within each experimental condition, and was reported.

Supplementary note

Nalfurafine at 10 μM did not bind significantly to 45 pharmacological targets, including histamine, neurokinin, bombesin, CGRP, somatostatin, ionotropic glutamate, dopamine, adrenergic, muscarinic, adenosine, IL-1β, IL-8, CCR1, CCR2, CCK, $GABA_A$, and VIP receptors and L-type Ca^{++} channel (Nakao and Mochizuki, 2009). The only target it exhibited a moderate affinity was the m1 muscarinic receptor with a K_i value of 1.7 μM (Nakao *et al*, 2009). Nalfurafine did not affect release of several inflammatory mediators (histamine, tumor necrosis factor, interleukin-1β and -6, and prostaglandins D2 and E2), nor did it influence activities of constitutive and inducible nitric oxide synthetase (Nakao *et al*, 2009).

Supplementary figures

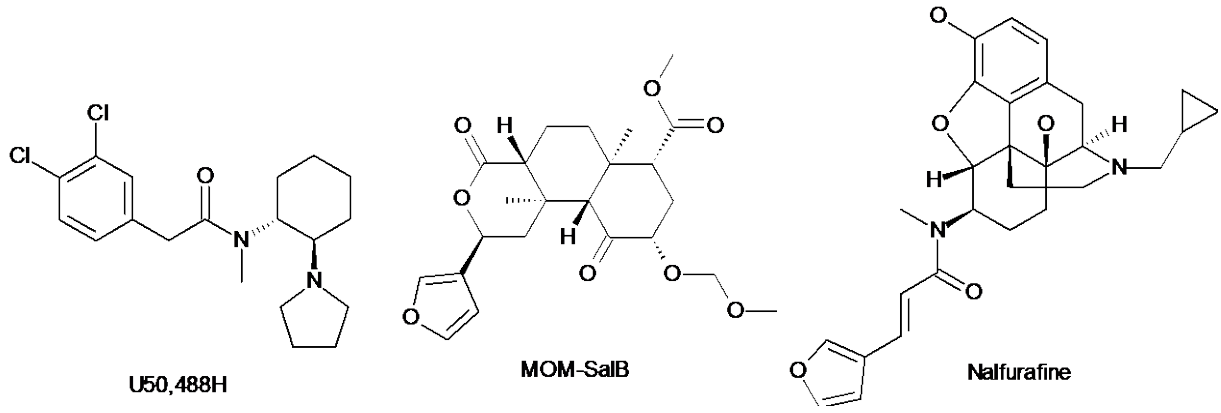

Figure S1. Chemical structures of U50,488H, MOM-SalB and nalfurafine

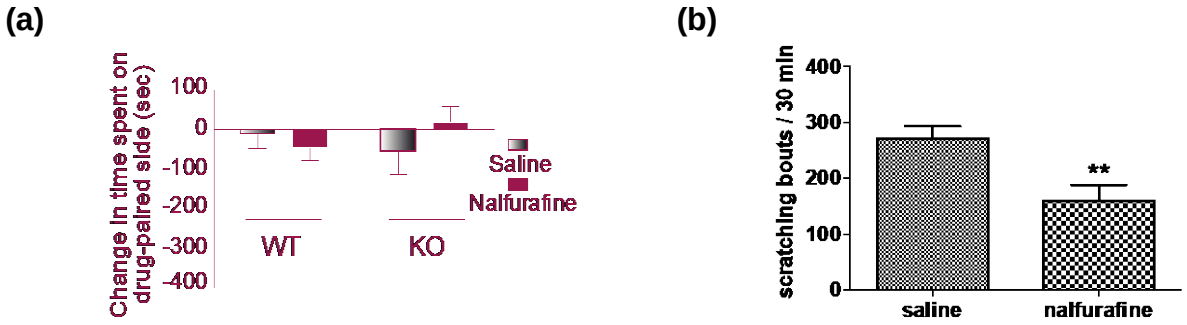

Figure S2. (a) Nalfurafine (10 µg/kg, s.c.) did not produce CPA in MOR-/- (KO) or wildtype (WT) mice. WT and KO mice in C57BL/6 background were conditioned and tested as described in Fig. 2c. Data are shown as mean ± SEM (n= 8-10). (**b) Nalfurafine (10 µg/kg, s.c.) inhibited compound 48/80-induced scratching by~40% in MOR-/- mice.** Experiments were performed as described as in Fig. 2a Data are shown as mean ± SEM (n=10). ** $p<0.01$ compared with saline by Student's *t* test.

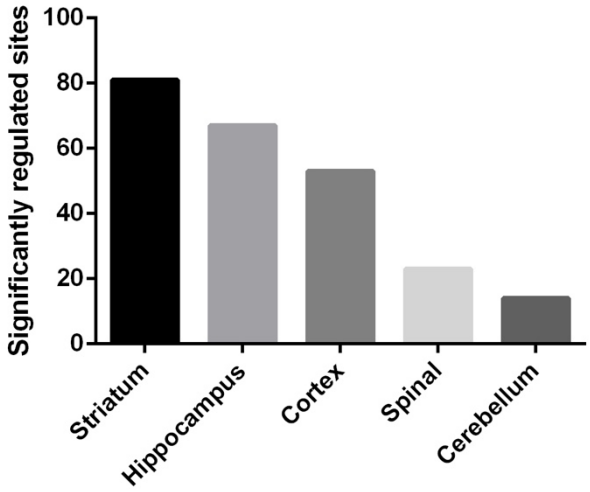

Figure S3. Numbers of significantly regulated sites in different brain regions: A two sample Student's t-test with a permutation-based False Discovery Rate (FDR) control was performed between U50,488H- and Saline-treated samples in respective brain regions. A relative stringent FDR < 0.2 cutoff was chosen to define "significantly regulated sites".

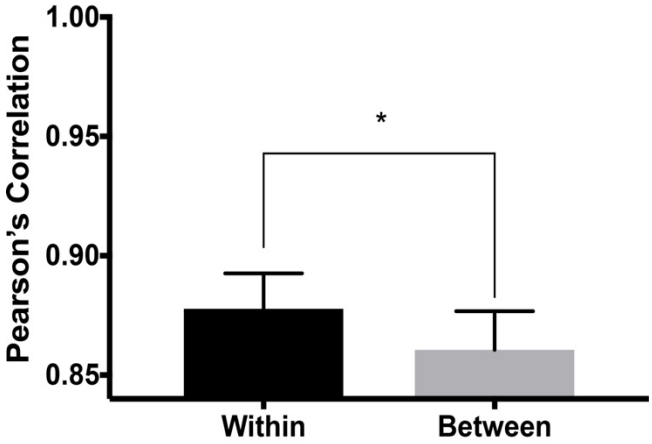

Figure S4. Comparison of Pearson's correlation of samples within each experimental condition (within) or across different experimental conditions (between). A Welch's t-test was performed, showing a statistically significant difference (p=0.024, unpaired, two-tailed Welch's t-test t=2.573, df=11.42).

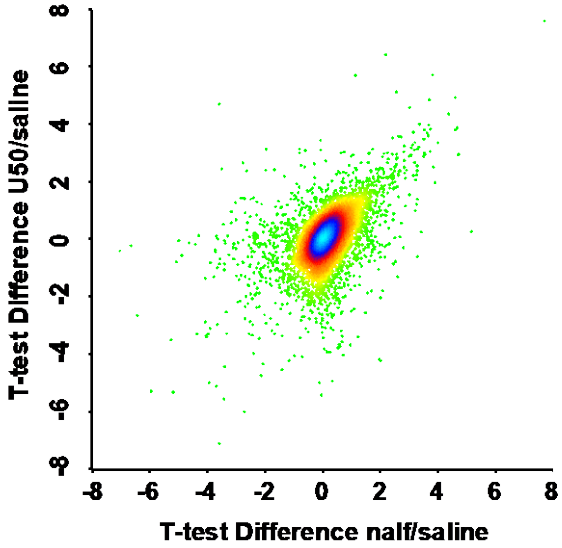

Figure S5. Plot of log2 differences in phosphopeptides obtained from two-sample student's t-test between either U50,488H (U50) or nalfurafine (nalf) treatment against saline treatment in the striatum. A density heat map was superimposed. Note that the vast majority of phosphorylation events was not changed by either treatment. Only ~5% phosphorylation events were perturbed by agonist treatment. Among them, most were similarly altered, whereas only a small percentage was differentially regulated.

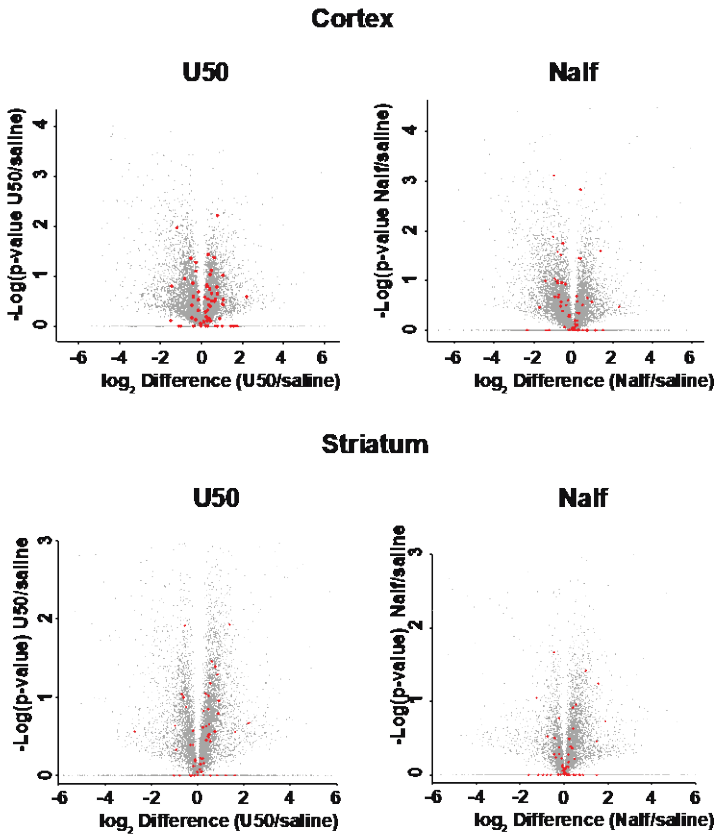

Figure S6. Volcano plots of Student's t-test results between U50,488H (U50) or nalfurafine (Nalf) treatment against saline treatment in the cortex and striatum. The background phosphosites were colored gray whereas the phosphosites annotated to proteins in mTOR pathway were colored red. A significant positive shift of distribution of mTOR phosphosites was observed in U50 samples, while no significant shift was observed in Nalf samples.

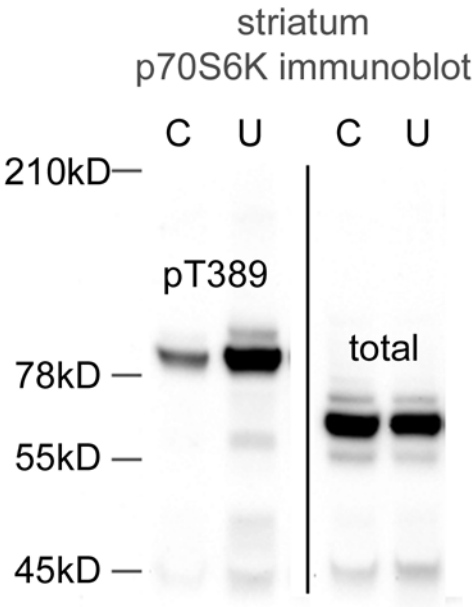

Figure S7. Effect of U50,488H on p70S6K phosphorylation at T389 in the mouse striatum. Male CD-1 mice were injected s.c. with saline or U50,488H (5 mg/kg) and killed 30 min later. Brains were removed and striata were dissected, weighed and homogenized in Laemmli sample buffer. Samples (40 μg protein/lane) were resolved in 8% SDS-PAGE and p70S6K phosphorylated at T389 and total p70S6K were detected by immunoblotting with specific antibodies as described in Methods. Images were captured with a Fuji LAS1000 plus system. Note that the Mr of the main protein band in the pT389 p70S6K blot is different from that of the main band in the total p70S6K blot. This blot represents one of the more than 5 experiments with similar results.
C: control; U: U50,488H.

Supplementary Tables

Table S1. Summary of behavioral data. A_{50} values were determined with the dose-response relationship plotted on a semi-log scale and analyzed using linear regression for the compound 48/80 test and the formalin test. Since all doses of U50,488H and MOM-SalB tested produced CPA, an A_{50} value could not be obtained for these two drugs. Nalfurafine is the only ligand tested that shows a separation in A_{50} values between aversion and antinociception/anti-scratching. ND: cannot be determined.

	Compound 48/80 test (anti-scratching)		**Formalin test (antinociception)**		**Conditioned place aversion (dysphoria)**	
	r^2	**A_{50}**	**r^2**	**A_{50}**	**r^2**	**A_{50}**
Nalfurafine	0.99	8.00 µg/kg	0.95	5.80 µg/kg	NA	ND
U50,488	0.99	2.07 mg/kg	0.98	0.58 mg/kg	NA	< 0.25 mg/kg
MOM-SalB	0.95	0.070 mg/kg	0.97	0.017 mg/kg	NA	< 0.001 mg/kg

Table S2. EC_{50} and E_{max} values of nalfurafine, U50,488H and MOM-SalB in promoting [^{35}S]GTPγS binding in mouse neuro2A cells stably transfected with the mouse KOPR (N2A-FmK6H cells)**.** Membrane preparation and KOR agonist-promoted [^{35}S]GTPγS binding were performed as we descried previously (DiMattio *et al*, 2015). Data are shown as mean ± SEM.

	pEC_{50} (EC_{50} mean, nM)	E_{max} (% of Enadoline)	n
U50,488	8.39 ± 0.06 (4.1)	94.9 ± 2.8	4
Nalfurafine	9.96 ± 0.04 (0.11)	111 ± 2.0	3
MOM-SalB	9.09 ± 0.06 (0.81)	97.1 ± 2.9	3

Table S3. EC_{50} and E_{max} values of nalfurafine, U50,488H and MOM-SalB in promoting β-arrestin recruitment *in vitro*. HEK293 cells stably transfected with the mouse KOPR (FmK6H) conjugated with β-galactosidase donor fragment and β-arrestin-1 or β-arrestin-2 fused with β-galactosidase acceptor fragment were used. Cells were grown in 96-well plates in MEM + 10% FBS to ~80% confluence. Different concentrations of a KOR agonist or vehicle was added and incubated at 37ºC in 5% CO_2 humidified incubator for 30 min. β-arrestin recruitment assay was performed with Galacto-Star System (Applied Biosystems, Bedford. MA). Following incubation, cells were rinsed with PBS once and lysis buffer was added at 10 μl/well and incubated for 10 min at room temp. Reaction buffer (100 μl/well) was added, mixed well and incubated for 30 min. Signals were measured with a plater reader (Envision 2104, Perkin Elmer) using the luminescence setting. Data are shown as mean ± SEM (n=3, each in triplicate).

	β-Arrestin-1		β-Arrestin-2	
	pEC_{50} (EC_{50} mean, nM)	E_{max} % of U50,488	pEC_{50} (EC_{50} mean, nM)	E_{max} % of U50,488
U50,488	6.83 ± 0.14 (147)	100 ± 7.0	7.44 ± 0.15 (35.9)	100 ± 7.7
Nalfurafine	8.58 ± 0.16 (2.64)	122 ± 8.5	8.85 ± 0.07 (1.40)	129 ± 4.7
MOM-SalB	7.82 ± 0.09 (15.3)	160 ± 4.7	8.32 ± 0.07 (4.74)	119 ± 4.4

Table S4. The mouse KOR: log $RA_{i\text{-}G}$, log $RA_{i\text{-}b1}$, log $RA_{i\text{-}b2}$, log ($RA_{i\text{-}G}/RA_{i\text{-}b1}$), log ($RA_{i\text{-}G}/RA_{i\text{-}b2}$) and bias factors. The parameters were estimated from the same agonist concentration-response curves that were used to estimate EC_{50} and E_{max} values (Table S2, Table S3) using the method described by Ehlert (2008). The prototypic selective KOR agonist U50,488H was designated as the "balanced" or standard reference ligand. The bias factor for a given ligand is defined as the ratio of the larger RA_i value ($RA_{i\text{-}G}$ or $RA_{i\text{-}I}$) divided by the smaller of the two.

G, G proteins; b1, β-arrestin 1; b2, β-arrestin 2.

G, b1 and b2 in front of the bias factor indicate G proteins-, β-arrestin 1-, and β-arrestin 2-biased, respectively.

	log $RA_{i\text{-}G}$	log $RA_{i\text{-}b1}$	$log\frac{RAi-G}{RAi-b1}$	Bias factor	log $RA_{i\text{-}b2}$	$log\frac{RAi-G}{RAi-b2}$	Bias factor
U50,488H	0	0	0	1	0	0	1
MOM-SalB	0.71 ± .087	1.19 ± .091	-0.48	b1, 3.02	0.96 ± .092	-0.25	b2, 1.78
nalfurafine	1.64 ± .074	1.84 ± .083	-0.2	b1, 1.58	1.52 ± .080	0.12	G, 1.32

References

Cox J, Mann M (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26**(12): 1367-1372.

Cox J, Mann M (2012). 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. *BMC Bioinformatics* **13 Suppl 16**: S12.

DiMattio KM, Ehlert FJ, Liu-Chen LY (2015). Intrinsic relative activities of kappa opioid agonists in activating Galpha proteins and internalizing receptor: Differences between human and mouse receptors. *Eur J Pharmacol* **761**: 235-244.

Ehlert FJ (2008). On the analysis of ligand-directed signaling at G protein-coupled receptors. *Naunyn Schmiedebergs Arch Pharmacol* **377**(4-6): 549-577.

Humphrey SJ, Azimifar SB, Mann M (2015). High-throughput phosphoproteomics reveals in vivo insulin signaling dynamics. *Nat Biotechnol* **33**(9): 990-995.

Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I*, et al* (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* **383**(6603): 819-823.

Murray CW, Porreca F, Cowan A (1988). Methodological refinements to the mouse paw formalin test. An animal model of tonic pain. *J Pharmacol Methods* **20**(2): 175-186.

Muschamp JW, Van't Veer A, Parsegian A, Gallo MS, Chen M, Neve RL*, et al* (2011). Activation of CREB in the nucleus accumbens shell produces anhedonia and resistance to extinction of fear in rats. *J Neurosci* **31**(8): 3095-3103.

Nakao K, Mochizuki H (2009). Nalfurafine hydrochloride: a new drug for the treatment of uremic pruritus in hemodialysis patients. *Drugs Today (Barc)* **45**(5): 323-329.

Sharma K, D'Souza RC, Tyanova S, Schaab C, Wisniewski JR, Cox J*, et al* (2014). Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. *Cell Rep* **8**(5): 1583-1594.

Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T*, et al* (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* **13**(9): 731-740.

Wang Y, Tang K, Inan S, Siebert D, Holzgrabe U, Lee DY*, et al* (2005). Comparison of pharmacological activities of three distinct k ligands (Salvinorin A, TRK-820 and 3FLB) on k opioid receptors in vitro and their antipruritic and antinociceptive activities in vivo. *J Pharmacol Exp Ther* **312**(1): 220-230.

White KL, Robinson JE, Zhu H, DiBerto JF, Polepally PR, Zjawiony JK*, et al* (2015). The G protein-biased kappa-opioid receptor agonist RB-64 Is analgesic with a unique spectrum of activities *in vivo*. *J Pharmacol Exp Ther* **352**(1): 98-109.

Xu W, Wang Y, Ma Z, Chiu YT, Huang P, Rasakham K*, et al* (2013). l-Isocorypalmine reduces behavioral sensitization and rewarding effects of cocaine in mice by acting on dopamine receptors. *Drug Alcohol Depend* **133**(2): 693-703.