Supplemental Material

Animals and housing. Wild-type (male) or DOR (male), MOR (male and female) and KOR (male and female) knock-out (for detailed description see below, and Supplemental Figures 1 and 2) C57BL/6 mice (male, 20-23g, Taconic) were individually housed in ventilated Plexiglas cages at ambient temperature (21ºC) in a room maintained on a reversed 12L:12D cycle (lights off at 10:00, lights on at 22:00). Food and water were provided ad libitum. The mice were given two weeks to acclimatize to the individual housing conditions and reverse light cycle before the start of the experiments. Mice were weighed weekly. All animal procedures were pre-approved by the Gallo Center Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were not deprived of food or water at any time.

Generation of knock-out mice. Mice were produced by Xenogen Biosciences under contract with the Ernest Gallo Clinic and Research Center. The mouse chromosome sequences were retrieved from the Ensembl database and used as a reference. Blood alcohol concentration (BAC) clones were used for generating the homologous arms, the knock-out region as well as the DNA probes for screening targeted events. The 5' homologous arms, 3' homologous arms, and knock-out regions were generated by RED cloning/gap repair. The fragments were cloned in the 3loxP3NwCD vector and were confirmed by restriction digestion and end sequencing. The final vector was obtained by standard molecular cloning. The final vectors contain the homologous arms, 3 loxP

sequences, and G418 and DTA cassettes. Putative homologous clones were selected by G418 resistance and DTA sensitivity and screened by DNA blot. Homologous recombinant ES clones were transfected with a Cre-recombinase plasmid. Type 1 (non-conditional) alleles, in which loxP sites 1 and 3 were recombined (see Supplemental Figure 1) and Type 2 (conditional) alleles, in which loxP sites 2 and 3 were recombined (see Supplemental Figure 1) were identified by PCR. For the generation of the knock-outs described in this study, C57/Bl6 blastocysts were generated from the Type 1 clones and implanted into C57/Bl6 mice. F1 progeny were genotyped for transmission of the mutant allele by PCR.

Limited access drinking paradigm. From Monday through Friday, mice (9 per group) were presented with a 2-bottle choice (water and 10% ethanol or 2% sucrose) for a 4 hour period (11:00 – 15:00) while in the dark cycle. Outside the 2-bottle choice period, all mice had unlimited access to water. All fluids were presented in 100-ml graduated glass cylinders with stainless-steel drinking spouts inserted through two grommets in front of the cage. Bottles were weighed to the nearest decigram (0.1 g) at the start and end of the 2-bottle choice period. The positions of the tubes containing water and ethanol were reversed daily to limit the effects of positional preference. Mice were trained for 3 weeks, during which their ethanol intake and preference stabilized (Supplemental Figure 3). After the training period mice were injected with drugs subcutaneously (s.c) on Friday, 30 minutes before the start of the 2-bottle choice period. Ethanol and water consumption were measured and compared to the average of the consumption on Tuesday, Wednesday and Thursday (baseline value). Mice did not receive drug or ethanol on Saturday or

Sunday and showed no long lasting drug effect on their drinking behavior the following week, in which the mice were treated with a higher dose of the drug or a different drug.

Locomotor activity assay. On the testing day, mice (n=8) were habituated to the locomotor boxes for 30 minutes prior to the behavioral assay. Mice were injected s.c. with vehicle (saline or 5% DMSO), 25 mg/kg TAN-67 or 6 mg/kg NTB and placed back in the locomotor boxes. Locomotor activity was assayed 30 minutes after injection for 4 hours using Accuscan Digipro activity monitors (Accuscan, Columbus, OH). All mice were tested during their light phase between the hours of 9:00 and 14:00. Mice were placed into custom-made acrylic boxes that were 21 × 21 cm and 28 cm tall. One acrylic box was used per single Accuscan monitor. Activity monitors were themselves housed inside sound-attenuating chambers (Med-Associates, St Albans, VT) equipped with lights and fans, both of which were turned on during the testing session. To eliminate variability due to handling and novelty, on the two days before the testing day, mice were habituated to the locomotor boxes for 30 minutes, injected with saline and placed in the locomotor box for an additional 30 minutes.

Blood alcohol concentration. In order to determine the basal BAC levels in mice trained to prefer ethanol, blood was drawn from trained mice (n=12) immediately after the 4 hour ethanol access period had ended. To determine the drug effect on BAC the blood was drawn from trained mice (n=9) on the Friday the drug was injected, immediately after the ethanol access period had ended. A 20-30 μl sample was taken from each mouse by submandibular bleeding. Blood alcohol concentration was measured. Blood samples were

precipitated in 3% perchloric acid (200 µl final volume) and stored at 4°C until analysis. Ethanol concentration was quantified by the alcohol dehydrogenase assay. Samples (20 µl) were incubated in duplicate in 1 ml of 0.5 M Tris-HCl buffer (pH 8.8) containing 5.5 µg/ml of alcohol dehydrogenase and 1.5 mM β-nicotinamide adenine dinucleotide (β-NAD) for 40 min at room temperature. Accumulation of β-NADH was measured by reading sample absorbance at 340 nm. The ethanol concentration in the samples was estimated by using a standard calibration curve, which was linear between 0.1-30 nM ethanol (r^2=0.99).

Ethanol uptake after intragastric gavage and intra peritoneal injection. To determine the effect of DOR subtype selective drugs on ethanol uptake and metabolism C57BL/6 mice were injected with saline or drug 30 minutes prior to an intragastric gavage (i.g., n=8) with 1.5 g/kg ethanol or to an intra peritoneal (i.p., n=5) injection with 2.5 g/kg ethanol. Blood was drawn from mice 60 minutes after the oral gavage, and 30 minutes and 150 minutes after the i.p. injection. BAC was determined as explained above.

Receptor biotinylation and serial immunoprecipitation. Cells expressing FLAG-MOR alone, HA-DOR alone or FLAG-MOR and HA-DOR together, which had been carefully matched for expression, were grown to 90% confluency in 10-cm dishes. Cells were washed twice in PBS and biotinylated with 0.3 mg/ml disulfide-cleavable biotin (Pierce, Rockford, IL) at 4°C for 30 minutes to selectively label receptors at the cell surface. Cells were rinsed twice with PBS and incubated in buffer containing 9 mg/ml iodoacetamide and 10 mg/ml BSA at 4°C for 20 minutes, and were then lysed in buffer (150 mM NaCl,

25 mM KCl, 10 mM Tris_HCl, 0.1% Triton X-100, pH 7.4) with added protease inhibitors (Complete, Roche, Basel, Switzerland) and cleared by centrifugation at 10,000 rpm (Eppendorf 5417R) for 10 minutes at 4°C. Receptor heterodimers and homomers were immunoprecipitated either from lysates generated from cells co-expressing MOR and DOR (Fig. 7A, DOR/MOR co-express) or from lysates generated from cells expressing only MOR and only DOR that were mixed together after lysis. Receptor heterodimers and homomers were detected as follows: cell lysates were incubated with 20 μl of anti-FLAG M2-conjugated Sepharose (Sigma) or non-conjugated protein G sepharose 4B (Invitrogen, Carlsbad, CA) for 16 hours at 4°C to remove MOR homomers and MOR/DOR heterodimers. The supernatant separated from this immunoprecipitation was then incubated with 20 μl of HA-conjugated affinity matrix (Covance) for 16 hours at 4°C (IP fraction) to remove DOR homomers. The FLAG immunoprecipate was then incubated with 0.5 mg/ml FLAG peptide (Sigma) for 30 minutes at 4°C to release receptor complexes into the supernatant. This supernatant was then immunoprecipitated with 20 μl of HA-conjugated affinity matrix (Covance) for 16 hours at 4°C (Co-IP fraction) to detect heterodimers (HA-DORs that had first co-immunoprecipitated with FLAG-MORs). All immunoprecipitates were extensively washed with immunoprecipitation buffer followed by two washes with 10 mM Tris, pH 7.5. Receptors were deglycosylated with PNGase (NEB, Beverly, MA) in 10 mM Tris, pH 7.5, for 1 h at 37°C, denatured with SDS sample buffer, and resolved by SDS PAGE. Blots were blocked in Blotto, incubated with Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) for 30 minutes and washed thoroughly. Blots were developed with

enhanced chemiluminescence reagents (ECL, Amersham Biosciences) to detect heterodimers and homomers.

[^{3}H]DPDPE binding studies. HEK 293 cells stably expressing DOR, MOR alone or expressing DOR and MOR together were homogenized in ice cold binding buffer (50 mM Tris, pH 7.4). For competition binding assays the cell homogenates were incubated at room temperature for 60 minutes with 0.01-10,000 nM ligand (TAN-67 or NTB) in the presence of 0.5-2 nM [^{3}H]DPDPE (22.5 Ci/mmol, Perking Elmer, Boston, MA) in a total volume of 200 µl. The incubations were stopped by rapid dilution with ice cold binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filterplates that had been pre-treated with 0.3% polyethyleneimine (Sigma). Filters were washed three times with binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

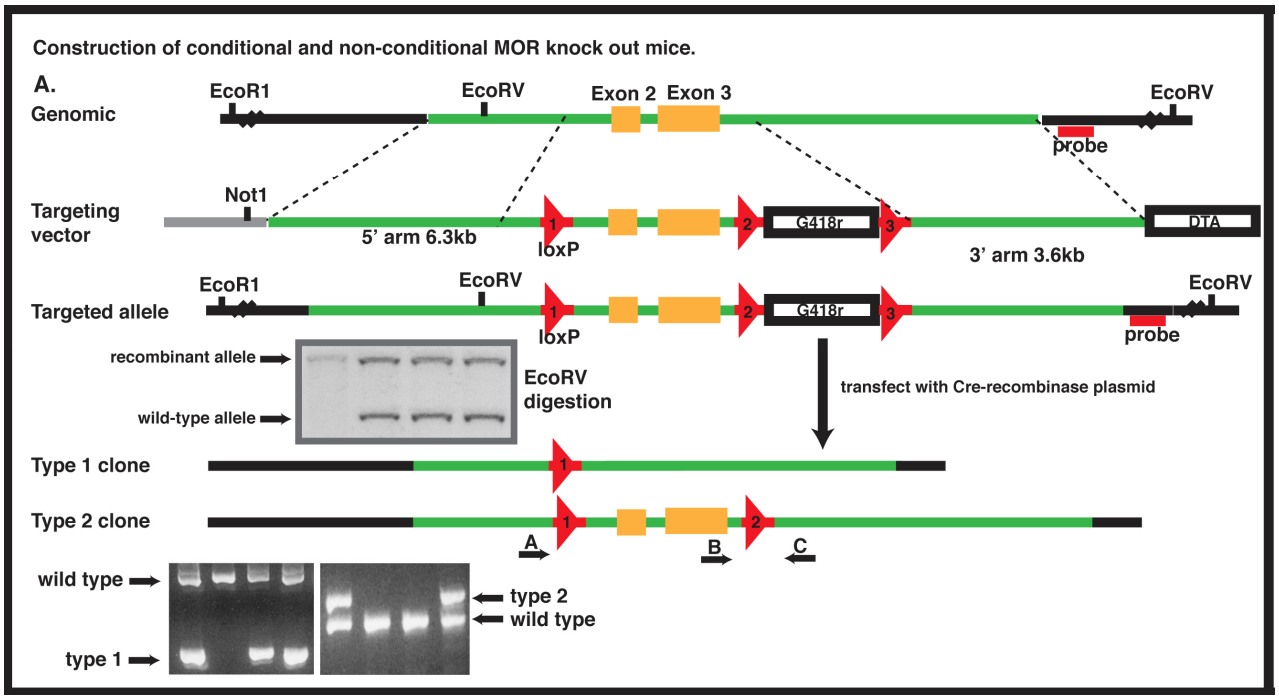
Calcium mobilization assay. HEK 293 cells stably expressing DOR, MOR or co-expressing DOR and MOR were maintained at 37ºC humidified in 7% CO_2/93% air atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum. Ca^{2+}-mobilization was measured in HEK 293 cells stably expressing the respective receptors that had been transiently transfected with chimeric G protein Δ6-Gqi4-myr (200 ng for every 60,000 cells). One day after transfection, cells were loaded for 60 minutes with a Ca^{2+}-fluorophore (Molecular Devices, Sunnyvale, CA) and stimulated with increasing amounts of ligand as indicated in the figure legends. Intracellular Ca^{2+}-release was measured immediately after agonist application in a Flex

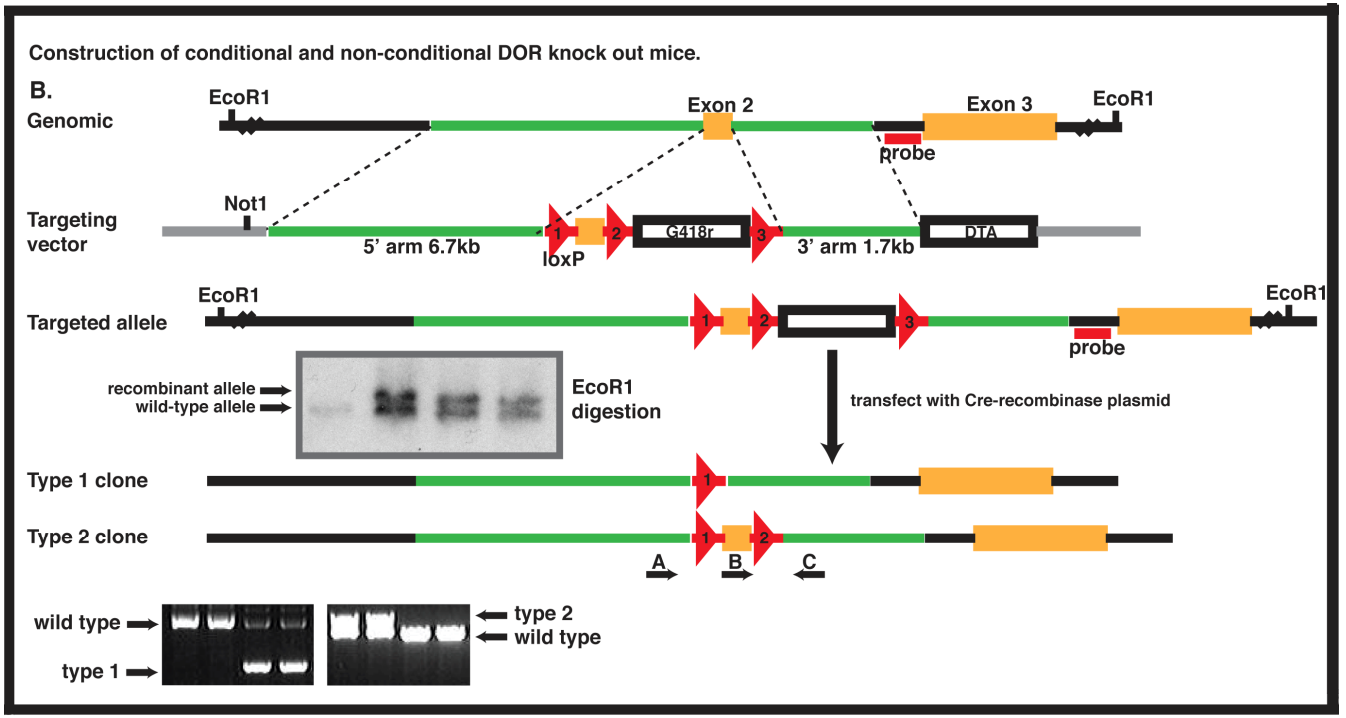
apparatus (Molecular Devices) for 2 minutes. When indicated cells were pre-incubated with antagonists 10 minutes prior to measurement of Ca^{2+}-release.

Conditioned place preference assay. Animals' place preference was monitored by the CPP apparatus, Opto-Max Activity Meter v2.27 (Columbus Instruments, Columbus, OH). The CPP cage, 42 × 20 × 20 cm, was separated by a removable guillotine door into two zones, with different color (black or white) and different floor texture. The apparatus was covered with a transparent Plexiglas lid perforated to allow adequate ventilation. The cage was equipped with matching pairs of horizontal sensors mounted alongside opposing lengths (42 cm long). The black and white zones (21 × 20 × 20 cm) were each scanned at a rate of 10Hz by 7 infrared beams, spaced at 2.54 cm intervals. On the first day, mice (n=12) were placed near the center of the CPP cage and allowed a 30 minute habituation period in which they were free to explore the black and white zones of the CPP apparatus. Time spent in the black and white zones was recorded for 30 minutes. Half the mice showed no preference, whereas the other half showed a slight preference for either the white or black zone. An unbiased approach was utilized, by pairing half the mice with drug on the preferred side and the other half with drug on the non-preferred side. During the conditioning phase (days 2–4) mice were injected twice daily with saline and drug or vehicle. Each injection was given 30 minutes before the animal was placed in the designated zone. Whether the mice would be administered drug in the morning or afternoon session was randomly assigned. Thus, each mouse received 3 TAN-67 or vehicle sessions and 3 saline sessions of 30 minutes each. During these sessions, mice were confined in either the black or white zone. On day 5, mice were again allowed to

freely explore the black and white zones of the CPP apparatus. Time spent in the black and white zones was recorded for 30 min. The induction of CPP was assessed by the difference in time it spent in drug-paired zone before and after conditioning.

Data analysis. Baseline values were determined by taking the average of the consumption over the three days prior to injection. Statistical analysis was performed using Prism software (GraphPad, San Diego, CA). Significance was determined by means of one-way ANOVA or two-way ANOVA (repeated measures). A post-hoc Newman-Keuls (one-way ANOVA) or Bonferroni (two-way ANOVA) test was used when a significant overall effect was found ($p<0.05$).


Construction of conditional and non-conditional MOR knock out mice.
A.
Genomic
EcoR1
EcoRV
Exon 2
Exon 3
EcoRV
probe
Targeting vector
Not1
5' arm 6.3kb
loxP
G418r
DTA
3' arm 3.6kb
Targeted allele
EcoR1
EcoRV
loxP
G418r
EcoRV
probe
recombinant allele
wild-type allele
EcoRV digestion
transfect with Cre-recombinase plasmid
Type 1 clone
Type 2 clone
A
B
C
wild type
type 1
type 2
wild type


Construction of conditional and non-conditional DOR knock out mice.
B.
Genomic
EcoR1
Exon 2
Exon 3
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probe
Targeting vector
Not1
5' arm 6.7kb
loxP
G418r
3' arm 1.7kb
DTA
Targeted allele
EcoR1
EcoR1
probe
recombinant allele
wild-type allele
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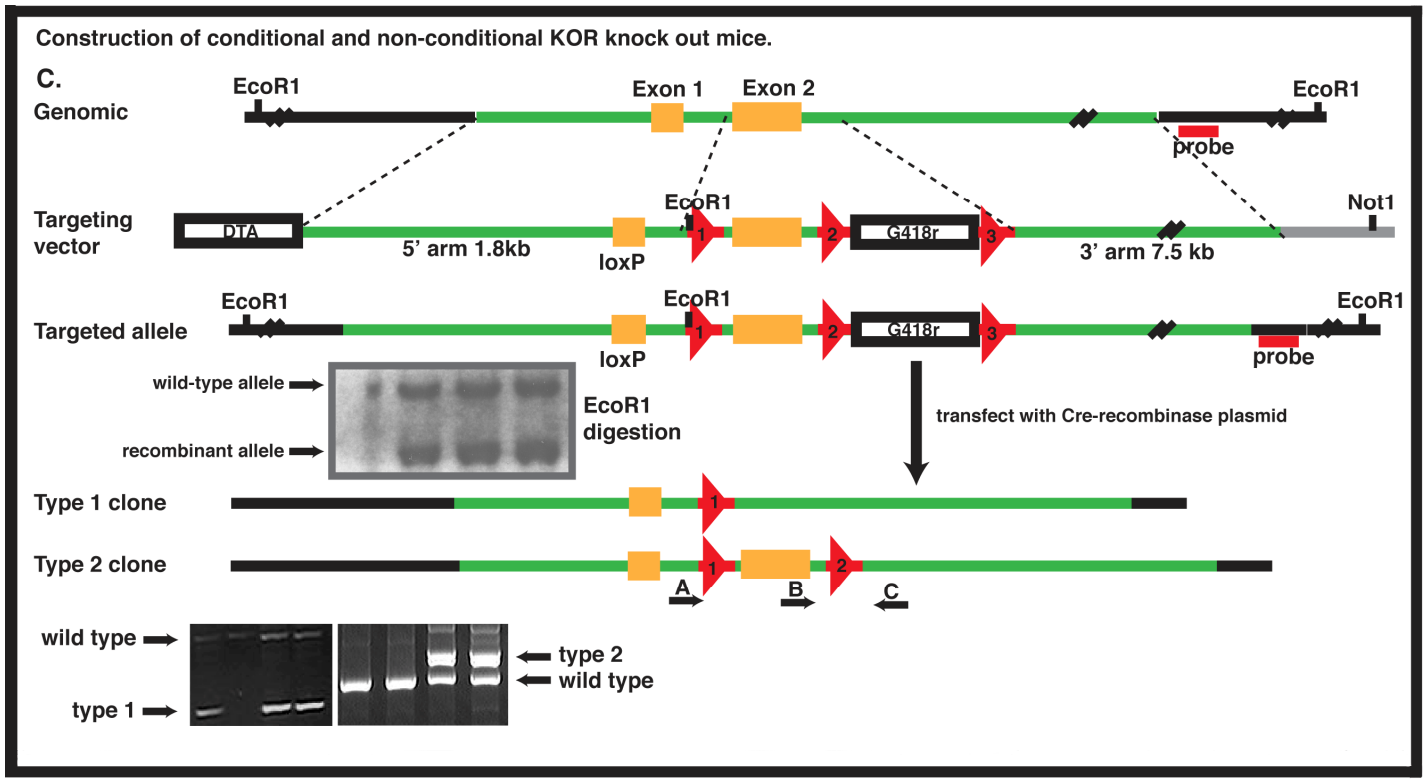
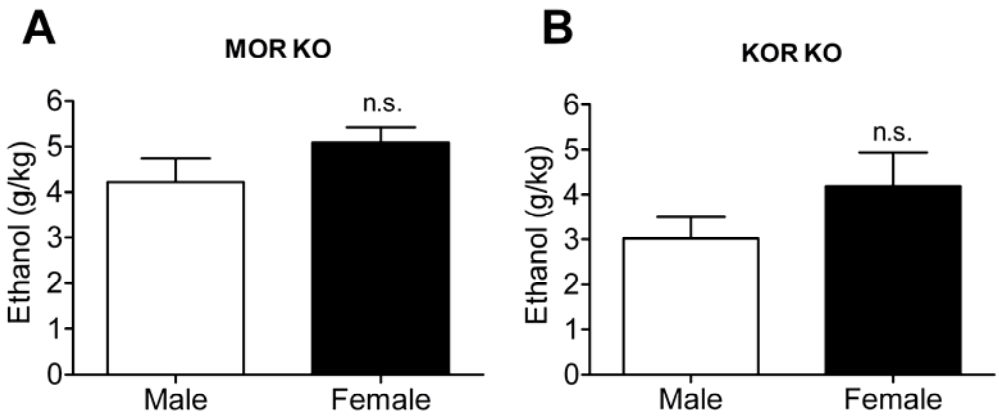
C
wild type
type 1
type 2
wild type

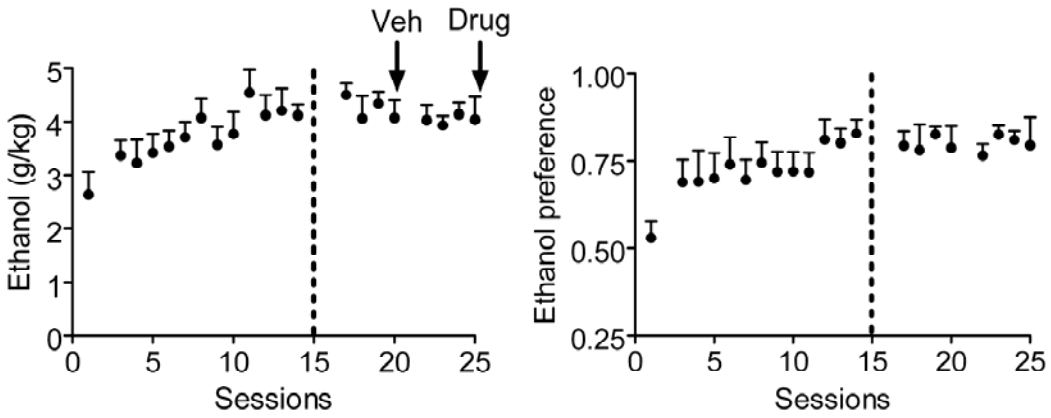


Supplemental Figure 1. Generation of opioid receptor knock-out mice. Mice were generated with non-conditional (type 1) and conditional (type 2) disruption of the MOR (**A**), DOR (**B**) and KOR (**C**) gene. The MOR type 1 mice are deleted for exon 2 and 3 and the type 2 mice have exons 2 and 3 flanked by loxP sites. The DOR type 1 mice are deleted for exon 2 and the type 2 mice have exon 2 flanked by loxP sites. The KOR type 1 mice are deleted for exon 1 and the type 2 mice have exon 1 flanked by loxP sites. Insets show DNA blots of homologous recombination events. These clones were transfected with Cre-recombinase and PCR screening of individual ES clones revealed type 1 (which produces non-conditional mutants) and type 2 (which produces conditional knock-outs) recombination events (lower insets). For this study, mice with a type 1 (non-conditional) allele were used.

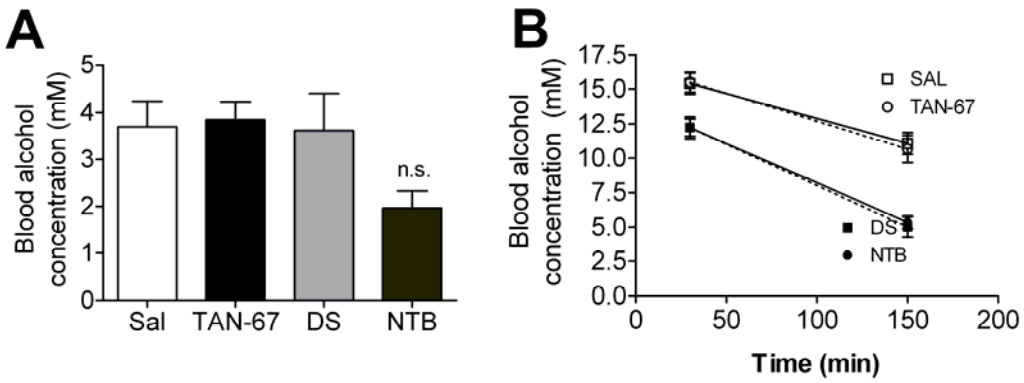


Supplemental Figure 2. No significant difference in ethanol consumption between male and female MOR and KOR KO C57BL/6 mice. Male (n=6) and female (n=3) MOR KO (**A**) and KOR KO (**B**) C57BL/6 mice (n=9) were trained to consume ethanol for three weeks. After training, basal ethanol consumption was measured for 3 days, over a 4 hour period and the average of these data are represented. Statistical significance was determined using an unpaired t-test (MOR KO $p = 0.28$; KOR KO $p = 0.20$). Also male and female subjects responded to drugs in similar fashion (data not shown).

n.s., non significant.

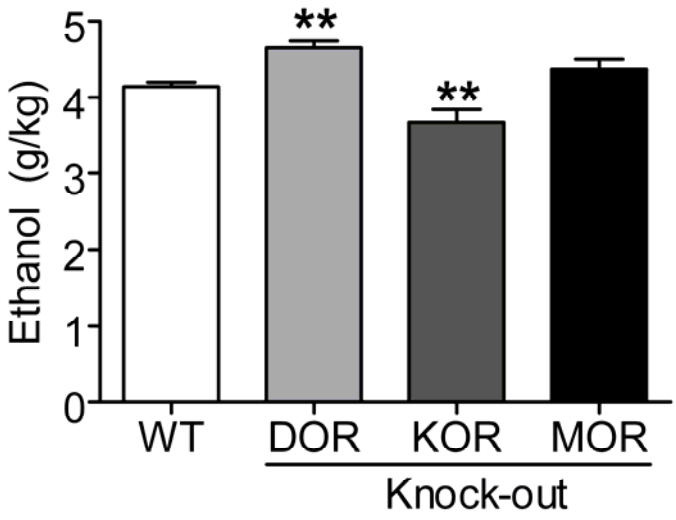


Supplemental Figure 3. Ethanol consumption and preference in C57BL/6 mice during training. Male wild-type C57BL/6 mice (n=9) were trained to consume ethanol for three weeks after which the consumption and preference reaches a stable plateau. Vehicle (Veh) or drug is injected on the first Friday after the training period. Mice are withheld from ethanol during the weekend during which the administered drug is cleared from the body. No persistent drug effect is seen on Monday and the following Friday a different dose or drug is injected.

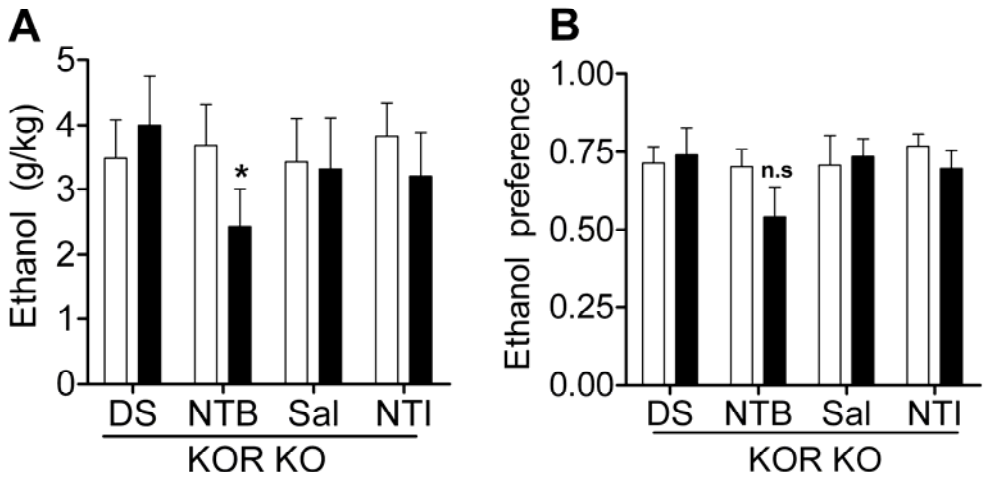


Supplemental Figure 4. TAN-67 and NTB do not significantly affect ethanol uptake or metabolism. (**A**) C57BL/6 mice were injected s.c. with vehicle (saline or 5% DSMO) 25 mg/kg TAN-67 or 6 mg/kg NTB. Thirty minutes after injection mice (n=8) were exposed to 1.5 g/kg ethanol by intragastric gavage. Blood alcohol levels (BAC) were measured 60 minutes after ethanol administration. (**B**) C57BL/6 mice (n=5) were injected s.c. with vehicle (saline or 5% DSMO, solid lines) or drug (25 mg/kg TAN-67 or 6 mg/kg NTB, dotted lines) 30 minutes prior to a 2.5 g/kg i.p. injection of ethanol. BAC was measured 30 minutes and 150 minutes post ethanol injection.

n.s., non significant.

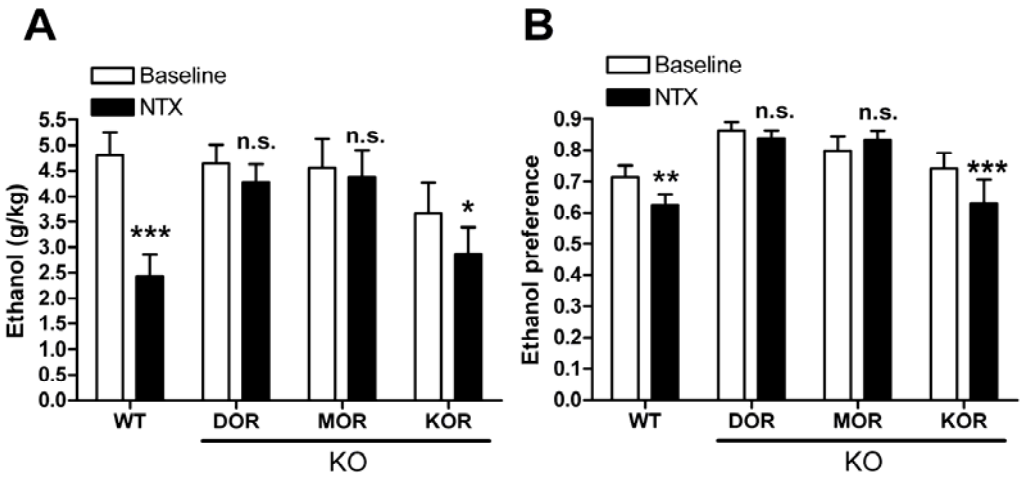


Supplemental Figure 5. Ethanol consumption in WT and opioid receptor knock-out mice. Wild-type, DOR KO, MOR KO and KOR KO C57BL/6 mice (n=9) were trained to consume ethanol for three weeks. After training, basal ethanol consumption was measured for 3 days, over a 4 hour period and the average of these data are represented. **p<0.01



Supplemental Figure 6. Disruption of KOR does affect NTI and NTB efficacy. Naltriben (NTB, 6 mg/kg) or naltrindole (NTI, 10 mg/kg) induced changes in ethanol consumption (**A**) and preference (**B**) in KOR KO C57BL/6 mice (n=9) trained to consume ethanol for three weeks. Thirty minutes after injection ethanol and water consumption was measured over a 4 hour period. Ethanol preference = ethanol consumption/(ethanol consumption + water consumption).

*p <0.05; n.s., non significant.



Supplemental Figure 7. Disruption of MOR, DOR and KOR affect NTX efficacy. Naltrexone (NTX, 1.5 mg/kg) induced changes in ethanol consumption (**A**) or preference (**B**) in wild-type, DOR KO, MOR KO and KOR KO C57BL/6 mice (n=9). Thirty minutes after injection (vehicle or drug) ethanol and water consumption was measured over a 4 hour period. Percentage decrease is ((Basal-drug)/Basal)x100. Ethanol preference = ethanol consumption/(ethanol consumption + water consumption). **p* <0.05; ***p*<0.01; ****p*<0.001; n.s., non significant.