Sex Differences in Sensitivity to the Depressive-Like Effects of the Kappa Opioid Receptor Agonist U-50488 in Rats

Supplemental Information

Supplementary Methods

Intracranial Self-Stimulation (ICSS)

Rats were anesthetized with sodium pentobarbital (65 mg/kg, IP; Abbott Laboratories, North Chicago, IL) supplemented with subcutaneous atropine (0.25 mg/kg) to minimize bronchial secretions and implanted with stainless steel monopolar electrodes (0.25 mm diameter; Plastics One, Roanoke, VA) aimed at the medial forebrain bundle at the level of the lateral hypothalamus (2.8 mm posterior to bregma, +1.7 mm lateral to midline, and 7.8 mm below dura). The electrodes were coated with polyamide insulation except at the tip. A non-insulated stainless steel wire was used as the anode and wrapped around a stainless steel screw embedded in the skull, and the entire assembly was coated with acrylic cement.

After one week of recovery from surgery, rats were trained to respond for brain stimulation using a continuous reinforcement schedule (FR1) at 141 Hz, where each lever press earned a 500 ms train of square wave cathodal pulses (100 ms per pulse), as described (1). The delivery of the stimulation was accompanied by illumination of a 2-watt house light. The stimulation current was adjusted (final range: 110 – 250 µA, with no significant difference between males and females, **Figure 1A**) for each rat to the lowest value that would sustain a reliable rate of responding (at least 40 responses per 50 s). After the minimal effective current was found for each rat, it was kept constant throughout training and testing.

Rats were then trained on series of 15 descending stimulation frequencies (141 – 28 Hz, in 0.05 $\log_{10}$ Hz increments), as described previously (2, 3). To characterize the functions relating response strength to reward magnitude (rate-frequency function), a least-squares line of best fit was plotted across the frequencies that sustained responding at 20, 30, 40, 50, and 60% of the maximum rate using customized analysis software. The stimulation frequency at which the line intersected the X-axis (theta 0) was defined as the ICSS threshold (see (1)). This is considered the theoretical point at which the stimulation becomes reinforcing. Rats were trained for an average of 3-4 weeks until mean ICSS thresholds remained stable (±10% for 4 consecutive days).

Castration Surgery

Male rats were anesthetized with a mixture of ketamine plus xylazine (80 mg/kg plus 12 mg/kg, i.p.; Sigma-Aldrich) for castration surgery. An isoflurane-oxygen vapor mixture was administered via nosecone as needed (e.g. if twitching was observed) during the procedure. A ventral transverse incision (~2.0 cm) was made at the midline above the scrotum and the subcutaneous tissues were pulled back. A 2% xylocaine solution was applied to the incision area to minimize pain. A small cut was made with a scalpel in the muscle surrounding the testes and surgical forceps were inserted and opened and closed to spread out the muscle and tissue in order to expose the testes. The testes were externalized, tied off with suture, and excised. The body wall and skin were each closed with sutures, and surgical staples were used to help protect the sutures. All wounds were treated with antibiotic ointment and rats were treated with ketoprofin (5.0 mg/kg, s.c.) for post-operative pain.

Female Estrous Cycle Tracking: Vaginal Cytology

To track female estrous cycles and ensure a normal 4-5 day cycle, vaginal smears were taken from female rats every morning at the same time for approximately two weeks before testing in any experiment. To control for the effects of daily handling, males were handled in a similar manner to the females. To obtain a vaginal swab, the tip of a 200 mL pipette was inserted into the vagina and 120 mL of distilled water was expelled and then immediately collected and placed on a glass microscope slide, as in (4). Slides were examined with a light microscope to obtain a rapid assessment of cytology and determination of cycle stage so that rats could be placed into treatment groups based on estrous cycle stage. Slides were then allowed to dry overnight and subsequently stained with hematoxylin and eosin Y. Briefly, slides were dipped in 95% ethanol and stained in Harris hematoxylin solution (Protocol, Kalamazoo, MI) for 2.5 minutes. They were then dipped several times in distilled water and placed in Scott's Tap Water Substitute (166 mM magnesium sulfate and 42 mM sodium bicarbonate) for one minute. The slides were dipped in distilled water and immersed in 95% ethanol for 30 seconds before being placed in Eosin Y (Sigma-Aldrich, St. Louis, MO) for 45 minutes. Slides were dipped sequentially in 95% and 100% ethanol until the slides were clear except for the stained cells. Finally, slides were placed in xylene for 10 minutes and coverslipped.

Vaginal cells were examined under the light microscope at 20x magnification (see **Figure S1**). The relative ratios of different cell types were used to determine the phase of the estrus cycle (4). In metestrous (6-8 hr phase), the ratio of leukocytes to other cells is about 10:1. In diestrous (55-57 hr phase), the ratio of leukocytes to other types of cells is 3:1. In

proestrous (12-hr phase), there are no leukocytes present, but rather uniform, nucleated, epithelial cells. In estrus (25-27-hr phase), the epithelial cells are completely cornified and clumped together. See **Figure S1**.

Repeated Blood Sampling for Pharmacokinetic Studies

Rats were cannulated through the right external jugular vein under 1.5-3% isoflurane-oxygen anesthesia (0.5 L/min flow rate). Rats were placed supine and the pulse was identified. For each rat, a small incision (1.5 cm) was made with a scalpel blade between the sternum and the right clavicle parallel to the long axis of the right jugular vein. Subdermal fat, connective tissue and muscle were displaced by the repeated opening and closing of a blunt-end scissors until the vein was clearly visible. The blood vessel was then loosened from its adhering tissue and small pieces of suture were placed under the veins at anterior and posterior ends and loosely tied. Using a pair of straight fine forceps, a small incision was made in the vein through which the cannula was inserted. The cannula was made of Silastic tubing (0.64 mm id) placed over Portex tubing (0.58 mm id), with a 12 mm overlap. A 2 mm Silastic collar was placed over the junction of Silastic and Portex tubing. After insertion, the cannula was tied to the vein, both at the point of insertion and also at a more anterior point on the vein. A small volume (50 µL) of sterile saline was injected and then an equal volume of blood was drawn into the cannula to check for patency. The line was subsequently cleared with saline and exteriorized subcutaneously at a point in the nape between the shoulder blades using a 19-gauge needle. Approximately 5 cm length of cannula was exposed to the exterior. The wound was then sutured using surgical thread. Sterile saline with heparin (0.5 mL of 50 U/mL) was injected into the cannula and the opening was finally blocked with a short pin. Each cannula was kept patent by flushing daily with 0.5 mL of heparinized saline.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

U-50488 concentrations in plasma and brain were determined by LC-MS/MS. Plasma and brain samples were stored at -80°C until processing. For plasma sample analysis, calibration curve standards were prepared in rat plasma at concentrations ranging from 0.0847 to 5000 ng/mL. Twenty-five µL of each plasma sample and calibration standard were extracted by adding 3 volumes of a mixture containing 0.1% formic acid and 2 µM tolbutamide (internal standard) in acetonitrile. For brain sample analysis, treated and blank rat brains were homogenized using a Power Gen 125 homogenizer equipped with a 10 x 95-mm homogenizer probe (Thermo Fisher Scientific) in 3 mL of the extraction solvent described above per g of

brain. Calibration curve standards were prepared in homogenates of blank rat brain at concentrations ranging from 0.0847 to 5000 ng per g of brain (one g of brain is equivalent to 1 mL). All samples were mixed by vortexing and centrifuged.

Samples were analyzed by multiple reaction monitoring on an LC-MS/MS system consisting of dual Shimadzu LC-10AD high-performance liquid chromatography pumps and a DGU-14A degasser (Shimadzu, Columbia, MD), a CTCPAL autoinjector (Leap Technologies, Carrboro, NC), and an API3000 or LC-MS/MS system, equipped with an electrospray ion source and operated by the Analyst software package (Applied Biosystems, Foster City, CA). Chromatography was conducted on a Sprite Armor C18 (20 x 2.1 mm, 5 µm) analytical column (Analytical Sales and Products, Pompton Plains, NJ) with a 0.5-m PEEK guard filter, at a flow rate of 0.55 mL/min using the following mobile-phase gradient program: mobile phase A (MPA) H_2O with 0.1% formic acid; mobile phase B (MPB) acetonitrile with 0.1% formic acid; 0 min 98% MPA, 2% MPB; 0.8 min 5% MPA, 95% MPB; 1.0 min 5% MPA, 95% MPB; 1.1 min 98% MPA, 2% MPB; 1.2 min end of run; approximately 1.5 min between sample injections. For the first 0.5 min of each sample run, the LC eluent was diverted from the ion source to waste.

Non-compartmental pharmacokinetic parameters for U-50488 in plasma were calculated using WinNonlin v5.1 (Pharsight Corp., St. Louis, MO).

Female Estrous Cycle Tracking: 17b-estradiol ELISA

Estradiol levels are lowest during estrous and gradually increase through metestrous and diestrous to peak during proestrous. To verify that actual estradiol levels corresponded to levels predicted from vaginal cytology, ELISA (E2 ELISA, Calbiotech, Inc) was used to measure plasma estradiol levels. The sensitivity of the kit is reported as <3 pg/ml. Plasma from the 0.083 hr post-U-50488 samples was used for determination of estradiol levels. Absorbance of the colorimetric reaction was read at 450 nm with a microplate reader and the unknowns were compared to a standard curve to calculate estradiol concentrations (see **Figure S1**). Data from each rat are grouped according to estrous cycle stage as determined by vaginal swab on the day of blood collection.

Immunohistochemistry (IHC)

Fos immunohistochemical staining

Rats were overdosed with pentobarbital (130 mg/kg, i.p.) and transcardially perfused with ice-cold 0.9% saline (NaCl) followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The fixed brains were removed and postfixed for 3 d at 4^{o}C, then

transferred to 20% glycerol in 50 mM phosphate buffer (PB; pH 7.4) at 4°C until saturation (≥24 hr). Coronal sections (30 µM) from the nucleus accumbens (NAc) shell and core, central nucleus and basolateral nucleus of the amygdala (CeA and BlA, respectively), paraventricular nucleus of the hypothalamus (PVN) and bed nucleus of the stria terminalis (BNST) were cut on a freezing microtome and stored in cryoprotectant (50% ethylene glycol, 20% glycerol, 10 mM PB, 150 mM NaCl, 3 mM KCl) at –20°C until IHC was performed. For c-Fos IHC, free-floating sections were rinsed 3 x 10 min in 0.01 M Tris-buffered saline, pH 7.4 (TBS) and then blocked for 2 hr at room temperature in AB media (0.3% Triton X-100 (TX), 2% normal goat serum (Invitrogen, Carlsbad, CA), and 1% bovine serum albumin (BSA, Sigma) in 0.01 M TBS). The sections were then incubated on a shaker overnight at room temperature with a polyclonal antibody made in rabbit directed against c-Fos (PC38T, Calbiochem, La Jolla, CA), diluted 1:10,000 in AB media. The following day, sections were rinsed 3 x 10 min in 0.01 TBS and incubated for 1 hr at room temperature in biotinylated goat anti-rabbit immunoglobulin G secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in AB media. Following 3 x 10 min rinses in 0.01 M TBS, sections were incubated with avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories) for 30 min at room temperature. After 3 x 5 min rinses in 0.01 M TBS, sections were reacted with 0.05% 3,3'diaminobenzidine tetrahydrochloride containing 0.01% H_2O_2 (DAB, Sigma) for 10 min. Rinsing in 0.01 M phosphate buffer terminated the reaction.

Quantification of c-Fos expression

To quantify the number of c-Fos-positive nuclei in brain regions of interest, still images were taken at 10x or 20´ magnification using a Zeiss Axioscope 2 (Zeiss, Oberkochen, Germany) and a digital camera (AxioCam, Zeiss) interfaced with a Macintosh G4 computer. Images were taken from 2-3 sections per treatment corresponding approximately to bregma +1.60 mm (NAc core and shell); -0.30 mm (BNST); -1.80 mm (PVN); -2.30 mm (amygdala, CeA and BlA) (6). Digital images were analyzed with Image J software for Macintosh (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/) by an observer unaware of the treatment groups. Each brain region of interest was outlined using anatomical markers (see **Figures 5, 6, and 7**). The area of the outlined region was measured using arbitrary units (pixels/inch) and was used to calculate the density of c-Fos staining in each section (density = number of c-Fos-positive nuclei/area). A threshold intensity and size range for c-Fos-positive nuclei was set so that all positively labeled cells in a region of interest were counted and signal due to background labeling was not. These parameters were determined separately for each experiment and were used for all analyses within an experiment.

Dual immunolabeling for Fos and corticotropin releasing factor

Two hours after U-50488 (10 mg/kg, i.p.) treatment, rats were overdosed with pentobarbital (130 mg/kg, i.p.) and transcardially perfused with ice-cold 0.9% saline (NaCl) followed by 2% paraformaldehyde, 0.2% picric acid, and 0.05% gluteraldehyde. Brains were post fixed for 24 hours then transferred to 20% glycerol in 50 mM PB; pH 7.4 at 4^{o}C until saturation (≥24 hr). Sections were sliced at 30 mm and stored in cryoprotectant until IHC was performed. Free-floating sections were rinsed 3 x 10 min in 0.1 M PB and endogenous peroxidase activity quenched in 0.75% hydrogen peroxide for 30 min followed by 3 x 10 min washes in 0.01 M PB. Tissue was then directly incubated in a-cFos (Calbiochem, 1:10,000) in 0.01 M phosphate-buffered saline, pH 7.4 (PBS) with 0.3% Triton X-100 (Sigma) and 0.04% BSA (PBS-Tx-BSA) for 24 hours at room temperature under gentle agitation. Next, the tissue was washed 3 x 10 min in PBS-Tx-BSA and incubated in biotinylated goat anti-rabbit secondary antibody (Vector; 1:400) for 90 minutes. Tissue was washed 3 x 10 min in PBS-Tx and incubated in avidin-biotin-peroxidase complex (Vectastain ABC, Vector) for 90 minutes at room temperature. Sections were washed 2 x 10 min in PBS-Tx and 1 x 10 min in 0.01 M PB. Tissue was developed in 0.05% DAB (Sigma) containing 0.01% hydrogen peroxide in PB for 10 minutes and rinsed in water to stop the reaction. The tissue was washed in 0.01 M PB then quenched in 0.75% hydrogen peroxide for 20 minutes followed by 3 x 10 min washes in PBS-Tx. Sections were incubated in polyclonal a-CRF made in goat (Santa Cruz, 1:1000) in PBS-Tx-BSA for 36 hours at room temperature under gentle agitation. Tissue was washed 3 x 10 min in PBS-Tx-BSA and incubated in biotinylated horse anti-goat secondary antibody (Vector; 1:400) for 90 min at room temperature. The tissue was washed 3 x 10 min in PBS-Tx then incubated in ABC for 90 minutes. It was then developed with 0.05% SG substrate (Sigma) containing 0.01% hydrogen peroxide in PB for 10 minutes. Sections were mounted on Fisher Superfrost slides.

Statistics

The linear mixed model with both fixed (sex or cycle and dose or time) and random (rat) effects was used because data could not be collected for all rats at all drug doses, times, or estrous cycle stages, thus leaving several missing values in the dataset that standard ANOVAs cannot handle without excluding all data from rats with missing data points. In all analyses using the linear mixed model, data from females were first analyzed separately to determine whether there was a significant effect of cycle. If no effect of cycle was observed, then a subsequent analysis was performed in which female data were aggregated (i.e. the levels within

sex are male and female). If estrous cycle did have a significant effect on the dependent variable, then a subsequent analysis was performed in which the levels of the Sex factor were male, female-estrous, female-diestrous, and female-proestrous.

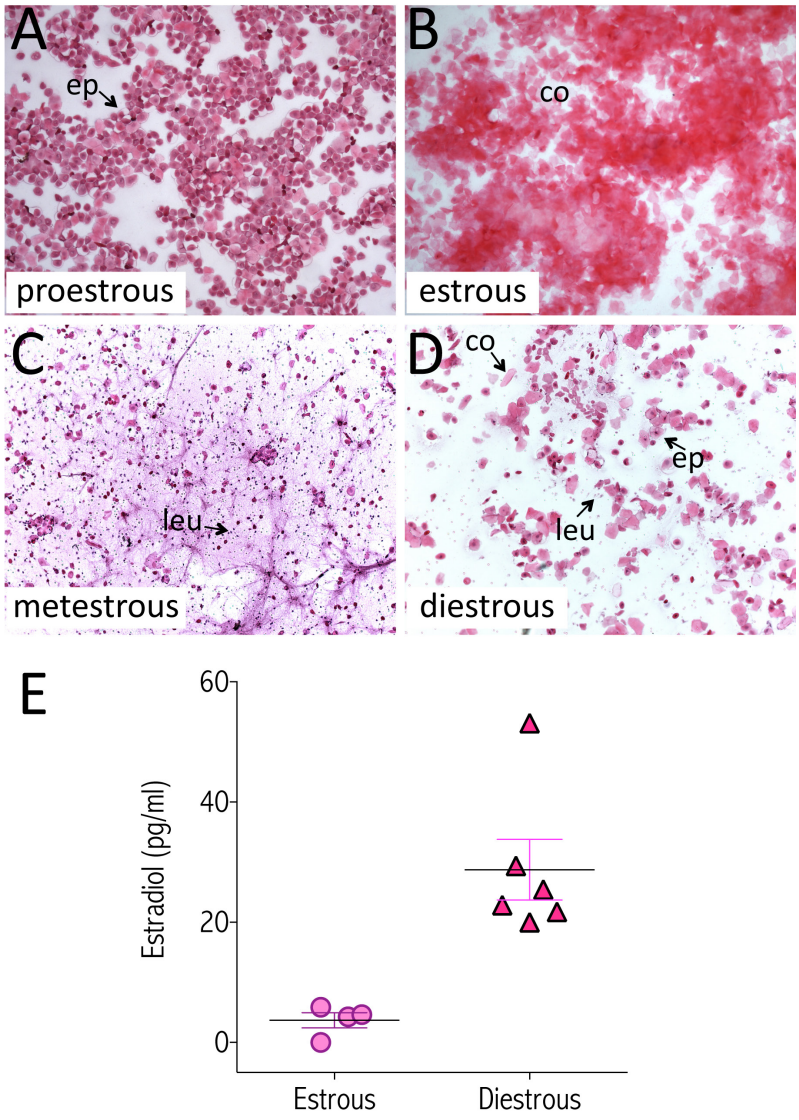

Figure S1. Vaginal smears were taken from female rats, applied to microscope slides, and subsequently stained with hematoxylin and eosin Y. Micrographs are representative of each stage and taken at 20x magnification. (**A**) Proestrous, a time of peak estradiol levels, is characterized by uniform, nucleated, epithelial cells (ep). (**B**) Estrous, a time post-ovulation when estradiol levels fall to low levels, is characterized by epithelial cells that are completely cornified (co) and clumped together. (**C**) Metestrous, the shortest phase (6-8 hr), is associated with low estradiol levels and the majority of cells are leukocytes (leu). (**D**) Diestrous, the longest stage (55-58 hr), is also characterized by leukocytes, but also contains substantial numbers of epithelial cells. (**E**) Plasma samples from 11 females rats used for the pharmacokinetic studies were also analyzed for 17β-estradiol using an ELISA. The estradiol levels for each rat (minus one rat whose plasma sample was inadvertently lost) are plotted against their predicted estrous cycles stage based on vaginal swab morphology, demonstrating that our method of estrous cycle tracking predicts with near perfect accuracy the actual estradiol levels of the female rats. The estradiol levels reported here match previously reported values (4).

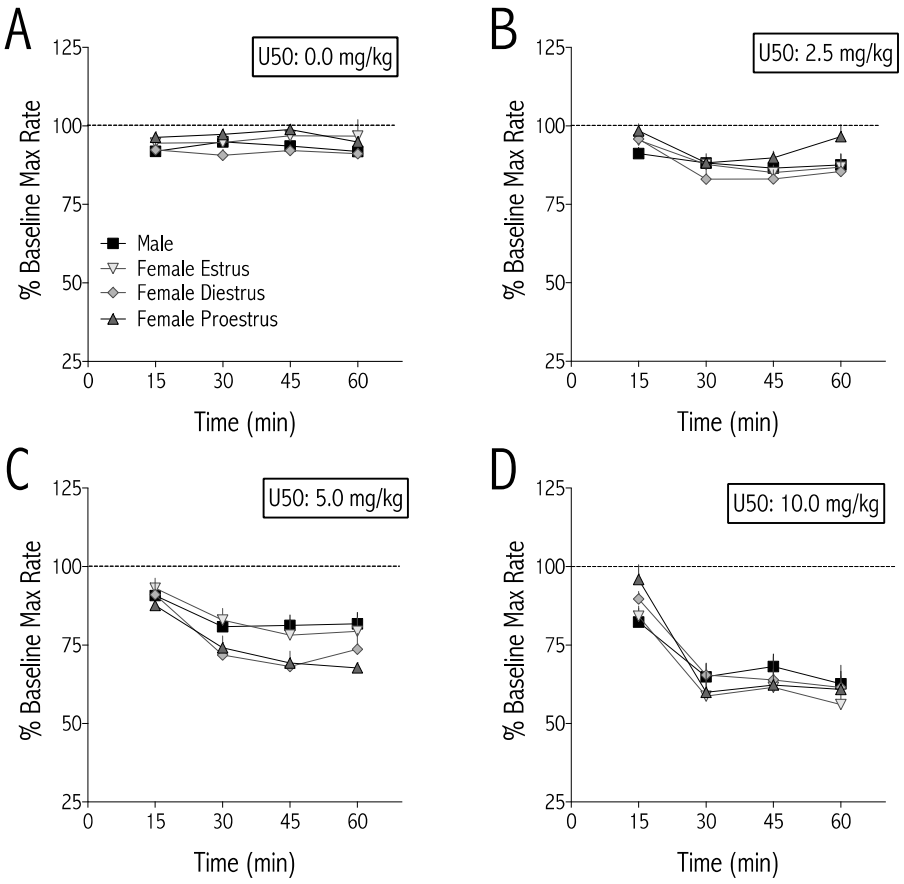

Figure S2. U-50488 decreases maximum rates of responding in male and female rats. U-50488 or vehicle (water) was administered (i.p.) right after baseline thresholds were determined and testing began immediately for one hour [Males (n = 9-13/dose), females (n = 7-13/dose/estrous cycle stage)] (mean ± SEM). The time course of effects of U-50488 on maximum rates of responding is displayed for each dose. The effects of U-50488 on maximum rates of responding depended on time (**A-D**) [2.5 mg/kg: $F_{(3,97)}$ = 4.770, p < 0.01; 5.0 mg/kg: $F_{(3,145)}$ = 13.054, p < 0.01; 10.0 mg/kg: $F_{(3,115)}$ = 26.626, p < 0.01], but not sex.

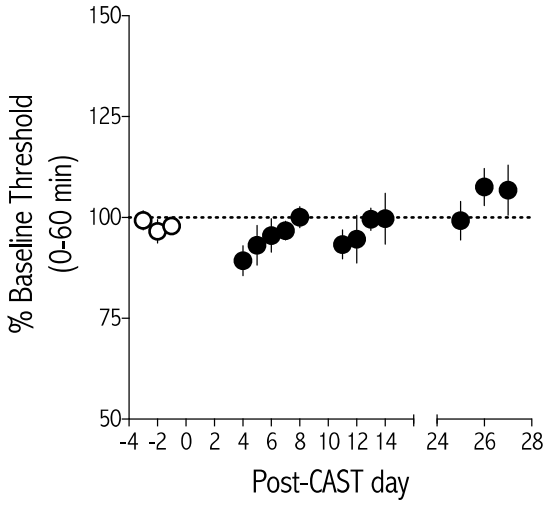

Figure S3. Four days after castration surgery, when rats had recovered sufficiently for behavioral analysis, ICSS thresholds were measured 5 days a week 23 days. Daily stimulation thresholds were compared to pre-castration baseline thresholds to generate % baseline threshold. Data from the days 4-14 and 25-27 post-castration are shown on the graph. There was no significant effect of castration on baseline ICSS thresholds over time, one-way ANOVA [$F_{(14,104)}$ = 1.99, not significant], although linear regression showed that the slope of the line through the data points is significantly non-zero [$F_{(1,103)}$ = 7.31, $p < 0.01$; $R^2 = 0.066$]. n = 7 rats. CAST, castration; ICSS, intracranial self-stimulation.

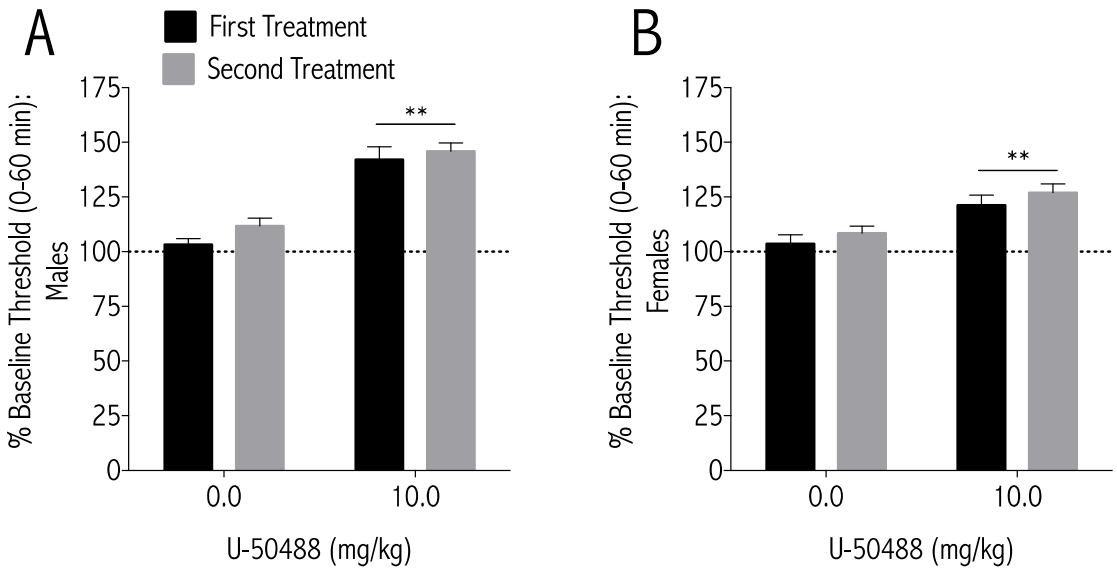

Figure S4. The effects of U-50488 (10.0 mg/kg) on intracranial self-stimulation thresholds are the same after the first and second treatments in both males and females, indicating no sensitization or tolerance. The percent change from baseline thresholds was compared after the first and second treatments. For each sex, the effects of U-50488 depended on dose: males [$F_{(1,11)} = 68.27$, $p < 0.01$] and females [$F_{(1,10)} = 13.90$, $p < 0.01$]. n = 12 males, 11 females.

Supplemental References

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