Effect of Chronic Delivery of the Toll-like Receptor 4 Antagonist (+)-Naltrexone on Incubation of Heroin Craving

Supplemental Information

Supplemental Methods

In Vitro Assays

Evidence from *in vitro* and *in vivo* studies indicates that (+)-naltrexone is a toll-like receptor 4 (TLR4) antagonist (1, 2). To further explore the selectivity of (+)-naltrexone and identify possible non-TLR4 sites of action, the compound was screened by Caliper Life Sciences for activity at 64 biologically-relevant sites including G protein-coupled receptors, nuclear receptors, ion channels and enzymes. In these assays, (+)-naltrexone was assessed in duplicate at 100 nM and 10 mM concentrations in parallel with positive control compounds. Greater than 50% inhibition was considered a "hit" (i.e., significant activity) by Caliper Life Sciences. Detailed information about the assay methods can be obtained on the company website.

As (-)-naltrexone displays high potency at the μ-opioid receptor (MOR) (3), we carried out our own investigation to directly compare the binding of (+) and (-) isomers of naltrexone at this receptor site; (-)-naltrexone HCl was obtained from Sigma and (+)-naltrexone HCl was synthesized by Kenner Rice. For binding assays, 3 nM [3H][D-Ala2-MePhe4,Gly-ol5]enkephalin ([^{3}H]DAMGO, SA = 44-48 Ci/mmol) was used as the radiolabel. On the day of the assay, Chinese hamster ovary (CHO) cells expressing human MOR were thawed on ice and homogenized with a polytron in 10 ml/pellet of ice-cold 10 mM Tris-HCl, pH 7.4. Homogenates were centrifuged at 30,000 x *g* for 10 min; pellets were then resuspended and centrifuged again at 30,000 x *g* for 10 min. Membranes were resuspended in 25^{o}C 50 mM Tris-HCl, pH 7.4 (~100 ml/pellet). All assays took place in 50 mM Tris-HCl, pH 7.4, with a protease inhibitor cocktail containing bacitracin (100 μg/ml), bestatin (10 μg/ml), leupeptin (4 μg/ml) and chymostatin (2 μg/ml) in a final assay volume of 1.0 ml. Drug dilution curves were made up with a buffer

containing 1 mg/mL bovine serum albumin, and non-specific binding was determined using 20 µM levallorphan. Triplicate samples were filtered with Brandel Cell Harvesters (Biomedical Research & Development Inc., Gaithersburg, MD), over Whatman GF/B filters, after a 2-h incubation at 25°C. The filters were punched into 24-well plates which contained 0.6 ml of liquid scintillation cocktail per well. Samples were counted, after overnight extraction, in a Trilux liquid scintillation counter at 44% efficiency.

Drugs for Behavioral Studies

Diacetylmorphine-HCl (heroin) and methamphetamine-HCl (methamphetamine) were provided by National Institute on Drug Abuse and dissolved in sterile saline. (+)-Naltrexone HCl.4$H_2$0 was synthesized by Kenner Rice and was dissolved in sterile water (chronic delivery) or sterile saline (acute injections). (+)-Naloxone HCl.2$H_2$0 was also synthesized by Kenner Rice and was dissolved in sterile saline (acute injections). The doses of (+)-naltrexone and (+)-naloxone were based on the previous study of Hutchinson *et al.* (4). These authors reported that at the dose range used in our study, acute injections of (+)-naloxone decreased intravenous remifentanil self-administration.

Subjects

Male Sprague-Dawley rats (Charles River, total *n* = 215), weighing 350–400 g prior to surgery, were used. The rats were group-housed (two per cage) prior to surgery and individually housed after surgery. The rats were maintained in the animal facility under a reverse 12:12 h light/dark cycle with food and water freely available. Procedures followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (8th edition; http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf). Thirty-five rats were excluded due to failure of catheter patency, health-related issues, or failure to acquire stable drug self-administration.

Intravenous Surgery

Rats were anesthetized with equithesin (9.72 mg/ml, 4 ml/kg intraperitoneal (i.p.)) or ketamine+xylazine HCl (75 mg/kg and 7.5 mg/kg, respectively, i.p.) and silastic catheters were inserted into their jugular vein, as described previously (5, 6). The catheter was attached to a modified 22-gauge cannula and mounted to the skull with dental cement. Buprenorphine (0.1 mg/kg, subcutaneous (s.c.)) was given after surgery to relieve pain and rats were allowed to recover for 5-7 days before heroin or methamphetamine self-administration training. During the recovery and training phases, catheters were flushed every 24–48 h with gentamicin (Butler Schein; 5 mg/mL) dissolved in sterile saline.

Minipump Implantation

In Exp. 1, 3, and 4, the osmotic minipumps (Alzet model 2ML2, Durect Corporation) were implanted s.c. in rats anesthetized with ketamine+xylazine (75:7.5 mg/kg, i.p), or isoflurane (4% induction; 2% maintenance) administered via a gas anesthesia machine with an attached scavenging device or hood. A small incision was made between the shoulder blades and the minipump was inserted under the skin. The incision was sutured and rats were given 2 ml of saline (injected s.c.) to replace fluid lost during surgery. The Alzet minipumps provided a constant infusion rate of 5-µl/h for 14 days and delivered either (+)-naltrexone or sterile water. (+)-Naltrexone was dissolved in sterile water. Calculation of (+)-naltrexone doses for the minipump delivery was based on mean body weight of the different dose groups (Exp. 1 or 3 heroin) or the mean body weight+25 g (Exp. 4, methamphetamine) on withdrawal day 1. The reported doses provide a higher estimate of the actual (+)-naltrexone dose that was delivered, because the rats in Exp. 1 (heroin) and Exp. 4 (methamphetamine) gained 74 ± 2 g (mean ± SEM n = 57) or 79 ± 4 g (n = 26) during the 13 withdrawal days. (+)-Naltrexone had no effect on weight gain (data not shown). Note: we used the mean body weight of the different groups for solution preparation, because we did not have a sufficient amount of (+)-naltrexone to make individual stock solutions (range of 75-85 mg/ml) for each rat.

Apparatus

The rats were trained in self-administration chambers, located inside sound-attenuating cabinets and controlled by a Med Associates (Georgia, VT) system. Each chamber had two levers located 8-9 cm above the floor. During self-administration training, presses on the retractable (active) lever activated the infusion pump (which delivered a heroin or methamphetamine infusion) or the pellet dispenser (which delivered a 45-mg food pellet, Cat. #1811155, Test Diet); presses on the stationary (inactive) lever were not reinforced with drug or food. Each rat's catheter was connected via a 22 gauge modified cannula (Plastics One) to a liquid swivel (Instech) via polyethylene-50 tubing that was protected by a metal spring.

Drug Self-Administration Training

The training procedure is based on our previous studies on incubation of cocaine, methamphetamine, and heroin craving (6-10). On the first training day, we brought the rats to the self-administration room where they were chronically housed in the chambers. We trained the rats to self-administer heroin or methamphetamine during three 3-h sessions per day (the sessions were separated by 1 h) under a fixed-ratio-1 with 20-sec timeout reinforcement schedule. The total number of training days was nine and they occurred over 9-10 days in Exp. 1-3a (heroin) or 13 days in Exp. 4 (methamphetamine) in order to prevent loss of body weight during the training phase [Note: under our training conditions, body weight for heroin-trained rats remains relatively stable, while the methamphetamine-trained rats lose about 4-8 g after each day of training]. Heroin and methamphetamine were dissolved in saline and self-administered at a dose of 0.1 mg/kg/infusion over 3.5 sec (0.10 ml/infusion) (8, 10, 11). During training, active lever-presses led to the delivery of a heroin or methamphetamine infusion and a compound 5-sec tone (2900Hz)-light (7.5-W white light located above the active lever) cue. The onset of each infusion was followed by a 20-sec timeout period, during which lever presses were recorded but not reinforced. The sessions started at the onset of the dark cycle and began with the extension of the active lever and the illumination of a red house light that remained on

for the duration of the session. At the end of each 3-h session, the house light was turned off, and the active lever was retracted.

Withdrawal Phase

During the withdrawal phase, the rats were housed in individual cages in the animal facility and were handled 3-4 times per week.

Extinction Tests

The experimental conditions during the extinction tests were the same as in training, except that presses on the previously active lever were not reinforced with drug. Tests started at the onset of the dark cycle and began with the extension of the active lever and the illumination of the red house light, which remained on for the duration of the session. Active lever responses during testing (the operational measure of cue-induced drug seeking in incubation of craving studies (12, 13)) resulted in contingent presentations of the tone-light cue that was previously paired with heroin or methamphetamine infusions, but not drug.

Exp. 1: Effect of chronic delivery of (+)-naltrexone during the withdrawal phase on incubated cue-induced heroin seeking

In Exp. 1 we determined the effect of chronic delivery of (+)-naltrexone during the withdrawal phase on incubated cue-induced heroin seeking, as assessed in 3-h extinction tests on withdrawal day 13. We trained 6 groups of rats (n = 8-10 per group, 3 vehicle groups, 3 drug-dose groups) to self-administer heroin for 9 days (see above) in three independent runs. Within each run (n = 18-20 per run), we tested the rats in a 30-min extinction session on withdrawal day 1 and then divided them into groups that received either chronic vehicle (sterile water in minipumps) or one of three chronic (+)-naltrexone dose conditions (7.5, 15, or 30 mg/kg/day); the vehicle and (+)-naltrexone rats were matched for extinction responding during this test, as well as heroin intake during training and mean body weight. Immediately after the 30-min extinction test, we implanted Alzet minipumps into the rats and brought them to the animal facility. On withdrawal day 13, we brought the rats back to the self-administration chambers and

tested them for cue-induced heroin seeking in a 3-h extinction session. We chose to assess incubated cue-induced heroin seeking on withdrawal day 13 for two reasons. The first is that cue-induced heroin seeking in extinction tests peaks after 1-2 weeks of withdrawal (7, 10). The second is that the duration of chronic drug delivery via the minipumps is 14 days, and we wanted to test the rats in the presence of (+)-naltrexone. [Note: in Exp. 1 and subsequent experiments, the duration of day 1 extinction test was only 30 min in order to minimize carry-over effect and extinction learning/experience during this test, which may decrease incubated cue-induced drug seeking on day 13 extinction test. We have incorporated this condition into our experimental design, because the within-subjects comparison in the vehicle group(s) between the first 30-min extinction responding on day 13 and the 30-min extinction test on day 1 provides empirical evidence for incubation of drug craving within each experiment].

Exp. 2: Effect of acute injection of (+)-naltrexone on incubated cue-induced heroin seeking

In Exp. 2 we determined whether acute injections of (+)-naltrexone prior to withdrawal day 13 extinction tests would mimic the effect of chronic delivery of (+)-naltrexone on incubated cue-induced heroin seeking. We trained 3 groups of rats (n = 10 per group) to self-administer heroin for 9 days; on the last 3 training days, we habituated the rats to the s.c. injection procedure. On withdrawal day 1, we injected all rats with sterile water (vehicle, 1 ml/kg, s.c.) and 10-15 min later tested them in a 30-min extinction session. After testing, we brought the rats to the animal facility. We divided the rats into 3 groups that were matched based on their extinction responding and heroin intake during training. On withdrawal day 13, we brought the rats back to the self-administration chambers and tested them for cue-induced heroin seeking in a 3-h extinction session. We injected the rats with vehicle (sterile water) or (+)-naltrexone (15 or 30 mg/kg, s.c.) 10-15 min before the late withdrawal extinction test.

Seven days after day 13 testing, 12 rats from Exp. 2 received surgically implanted minipumps containing sterile water (vehicle, n = 6) or (+)-naltrexone (30 mg/kg/day, n = 6) to determine the effect of (+)-naltrexone on high-rate operant responding for food. We performed

this test to rule out the possibility that (+)-naltrexone's effect on cue-induced drug seeking is due to non-specific sedative or other performance disrupting effects. Five days after the minipump implantation, we trained the rats to lever-press for food pellets (14, 15). We gave the rats two 1-h sessions (separated by 3 h) of 'autoshaping' (pellets were delivered non-contingently every 2 min into a receptacle and were accompanied by a compound tone-light cue). We then trained the rats to lever press for food pellets on a FR-1 20-sec timeout reinforcement schedule in two 1-h daily sessions (separated by 3 h) for 4 days; the first daily session started at the onset of the light-dark cycle and pellet delivery was accompanied by a 5-sec tone-light cue. The rats were given free access to regular food in their home cage after the daily sessions. Water was available in both the self-administration chambers and the home cages.

Exp. 3: Effect of chronic delivery or acute injections of (+)-naltrexone on heroin self-administration

In Exp. 1 we found that chronic delivery of (+)-naltrexone during the withdrawal phase decreased incubated cue-induced heroin seeking on day 13. To further assess the role of TLR4 in heroin-taking behavior, we determined whether chronic delivery or acute systemic injections of (+)-naltrexone would decrease ongoing heroin self-administration during the training phase. In the acute experiment, we also compared the effect of (+)-naltrexone (injected i.p. or s.c.) on heroin self-administration to the effect of (+)-naloxone, previously shown to decrease reminfentanil self-administration (4).

Exp. 3a. Chronic (+)-naltrexone delivery: Three groups of rats (*n* = 8-10 per group) received surgically implanted minipumps that delivered either sterile water (vehicle) or (+)-naltrexone (15 or 30 mg/kg/day); the surgeries occurred 2 days prior to the start of heroin self-administration training. We then trained the rats to self-administer heroin as described above.

Exp. 3b. Acute (+)-naltrexone or (+)-naloxone injections: We trained rats (*n* = 11) to self-administer heroin (as described above) and habituated them to the s.c. and i.p. injections prior to the start of testing, which began after 8 training days. Testing was similar to training in that

we gave the rats access to three 3-h daily sessions that were separated by 1 h. We tested the rats in a series of four repeated tests (separated by 1-2 days of regular training without injections) for the effects of (+)-naltrexone (0, 15 and 30 mg/kg, i.p. and s.c.) and (+)-naloxone (0, 15 and 30 mg/kg, i.p. and s.c.). We counterbalanced both the order of the 4 tests and the vehicle and the 2 doses of the TLR4 antagonists within each test. We injected the vehicle (sterile saline), (+)-naltrexone, and (+)-naloxone 10-15 min before the start of the test sessions at the onset of the dark cycle. One rat was not tested in the (+)-naltrexone s.c. condition because of loss of catheter patency.

Exp. 4: Effect of chronic delivery of (+)-naltrexone during the withdrawal phase on incubated cue-induced methamphetamine seeking

In Exp. 1 we found that chronic delivery of (+)-naltrexone during the withdrawal phase decreased incubated cue-induced heroin seeking. In Exp. 4 we determined the generality of this effect to incubation of psychostimulant (methamphetamine) seeking. The experimental conditions were similar to those described in Exp. 1, except that during self-administration training, lever-presses were reinforced with methamphetamine (0.1 mg/kg/infusion). Additionally, in order to maintain stable body weight during training, the rats were given four intermittent off days during the training phase to prevent loss of body weight (the total duration of the training phase was 13 days). During the extinction test on withdrawal day 13, one rat was tested 3 h later than the other rats due to a procedural error.

Statistical Analyses

MOR binding data were analyzed with MLAB-PC (Civilized Software) using a one binding site model to determine equilibrium dissociation constants (K_i) for displacement of [^{3}H]DAMGO. The behavioral data were analyzed with analyses of variance or covariance (ANOVA or ANCOVA) (Proc GLM, SPSS 15 or SAS 9.2). Significant effects from the factorial ANOVAs or ANCOVAs ($p < 0.05$) were followed by post-hoc pairwise comparisons between (+)-naltrexone dose groups in which the *p*-values were corrected for false-discovery rate (FDR) (16) using

SAS's Proc Multitest (Alpha level was set at 0.05 [FDR-corrected], two-tailed). The dependent measures were the number of infusions/pellets earned (training phase) and the number of active and inactive lever-presses (training and test phases); analyses were performed separately for infusions and lever (active, inactive). For the acute systemic injections of (+)-naltrexone and (+)-naloxone, (Exp. 3), we analyzed the data separately for each drug and route of administration (s.c. or i.p.). In all drug experiments except Exp. 3, we verified, in the vehicle-treated groups, that incubation of drug craving had occurred by assessing within-subjects increases in responding on the previously active lever during the 30-min extinction test on withdrawal day 1 and the first 30 min of the 3-h extinction test on day 13. In Exp. 1, the vehicle rats in the 3 runs (n = 8-10 per run) did not statistically differ in their active-lever extinction responding on withdrawal day 13 ($p > 0.05$); thus, their data were pooled to form a single vehicle group (n = 28) for the statistical analyses. The rats in the different chronic (+)-naltrexone conditions were matched for their drug intake during training and for extinction responding on withdrawal day 1. Thus, non-significant group differences during training and withdrawal day 1 are not reported. Additionally, for clarity, we only report main effects and interactions that are critical for data interpretation and indicate post-hoc group differences as asterisks in the figures. The specific within- and between-subjects factors in the different ANOVAs are provided in the Results section.

Table S1. Effects of (+)-naltrexone on radioligand binding and enzyme inhibition at selected targets. Ki is the equilibrium constant for inhibition of radioligand binding or inhibition of enzyme activity.

Target Protein	Species	Radioligand	*K_i
G Protein-Coupled Receptors			
Adenosine, Non-selective	Bovine	[^{3}H]NECA	>10 µM
Adrenergic, α1, Non-selective	Rat	[^{3}H]7-MeOxy-prazocin	>10 µM
Adrenergic, α2, Non-selective	Rat	[^{3}H]RX 821002	>10 µM
Adrenergic, ß1	Human	[^{125}I](-)-Iodocyanopindolol	>10 µM
Angiotensin II, AT1	Human	[^{125}I](Sar1-Ile8)Angiotensin	>10 µM
Angiotensin II, AT2	Bovine	[^{125}I]Tyr4-Angiotensin	>10 µM
Bradykinin, BK2	Guinea Pig	[^{3}H]Bradykinin	>10 µM
Cannabinoid, CB1	Human	[^{3}H]CP 55940	>10 µM
Cannabinoid, CB2	Human	[^{3}H]CP 55940	>10 µM
Cholecystokinin, CCK1	Mouse	[^{125}I]CCK-8	>10 µM
Cholecystokinin, CCK2	Mouse	[^{125}I]CCK-8	>10 µM
CRF, Non-selective	Rat	[^{125}I]Tyr0-oCRF	>10 µM
Dopamine, D4.2	Human	[^{3}H]YM-09,151-2	>10 µM
Endothelin, ETA	Human	[^{125}I]Endothelin	>10 µM
Endothelin, ETB	Human	[^{125}I]Endothelin	>10 µM
GABA-B	Rat	[^{3}H]CGP 54626A	>10 µM
Galanin, Non-selective	Rat	[^{125}I]Galanin	>10 µM
Glutamate, mGluR1	Rat	[^{3}H]Quisqualic acid	>10 µM
Glutamate, mGluR5	Rat	[^{3}H]MPEP	>10 µM
Histamine, H1	Bovine	[^{3}H]Pyrilamine	>10 µM
Histamine, H2	Guinea pig	[^{125}I]Aminopotentidine	>10 µM
Histamine, H3	Rat	[^{3}H]N-a-MeHistamine	>10 µM
Leukotriene, LTB4	Guinea pig	[^{3}H]LTB4	>10 µM
Leukotriene, LTD4	Guinea pig	[^{3}H]LTD4	>10 µM
Muscarinic, M1	Human	[^{3}H]N-Methylscopolamine	>10 µM
Muscarinic, M2	Human	[^{3}H]N-Methylscopolamine	>10 µM
Muscarinic, M (Central)	Rat	[^{3}H]QNB	>10 µM
Muscarinic, M (Peripheral)	Guinea pig	[^{3}H]QNB	>10 µM
Neurokinin, NK1	Rat	[^{3}H]Substance P	>10 µM
Neurokinin, NK2 (NKA)	Human	[^{125}I]NKA	>10 µM
Neurokinin, NK3 (NKB)	Rat	[^{125}I]Eledoisin	>10 µM
Opioid, Mu	Human	[^{3}H]Diprenorphine	>10 µM
Opioid, Kappa 1	Guinea pig	[^{3}H]U69593	>10 µM
Oxytocin	Rat	[^{3}H]Oxytocin	>10 µM
Platelet Activating Factor	Rabbit	Hexadecyl-[^{3}H]-acetyl-PAF	>10 µM

Target Protein	Species	Radioligand	*K_i
Thromboxane, TXA2	Human	[^{3}H]SQ 29,548	>10 µM
Thyrotropin Releasing Hormone	Rat	[^{3}H](3MeHis2)TRH	>10 µM
Vasoactive Intestinal Peptide	Rat	[^{125}I]VIP	>10 µM
Vasopressin, V1	Rat	[^{3}H]Phenyl-3,4,5-8-Arg-VP	>10 µM
Nuclear Receptors			
Estrogen	Human	[^{3}H]Estradiol	>10 µM
Glucocorticoid	Human	[^{3}H]Dexamethasone	>10 µM
Testosterone, Cytosolic	Human	[^{3}H]Methyltrienolone	>10 µM
Other Receptors			
Nitric Oxide, NOS (Neuronal)	Rat	[^{3}H]NOARG	>10 µM
Ion Channels			
Calcium, Type L (Benzothiazepine Site)	Rat	[^{3}H]Diltiazem	>10 µM
Calcium, Type L (Dihydropyridine Site)	Rat	[^{3}H]Nitrendipine	>10 µM
Calcium, Type N	Rat	[^{125}I]Conotoxin GVIA	>10 µM
GABA, GABA-A Agonist Site	Bovine	[^{3}H]GABA	>10 µM
GABA, GABA-A BDZ Alpha-1 Site	Bovine	[^{3}H]Flunitrazepam	>10 µM
Glutamate, AMPA Site	Rat	[^{3}H]AMPA	>10 µM
Glutamate, Kainate Site	Rat	[^{3}H]Kainic Acid	>10 µM
Glutamate, NMDA Agonist Site	Rat	[^{3}H]CGP 39653	>10 µM
Glutamate, NMDA PCP Site	Rat	[^{3}H]TCP	>10 µM
Glutamate, NMDA Glycine, Strych-insen	Rat	[^{3}H]MDL-105,519	>10 µM
Glycine, Strychnine-sensitive	Rat	[^{3}H]Strychnine	>10 µM
Nicotinic, α-BnTx Sensitive (Muscle)	Human	[^{3}H]α-Bungarotoxin	>10 µM
Nicotinic, α-BNtx Insensitive (Neuronal)	Human	[^{3}H]Epibatidine	>10 µM
Potassium, ATP Sensitive (KATP)	Rat	[^{3}H]Glibenclamide	>10 µM
Potassium, Ca^{++} Activated (VI)	Rat	[^{125}I]Apamin	>10 µM
Sodium, Site 2	Rat	[^{3}H]Batrachotoxin	>10 µM
Enzymes			
Glutamic Acid Decarboxylase GAD	Rat	[^{14}C]Glutamic Acid	>10 µM
Acetylcholinesterase	Human	Acetylthiocholine	>10 µM
Monoamine Oxidase A (Peripheral)	Rat	[^{14}C]5-HT	>10 µM
Monoamine Oxidase-B (Peripheral)	Rat	[^{14}C]Phenylethylamine	>10 µM
Choline Acetyl Transferase	Rat	[^{14}C]Acetyl Coenzyme	>10 µM

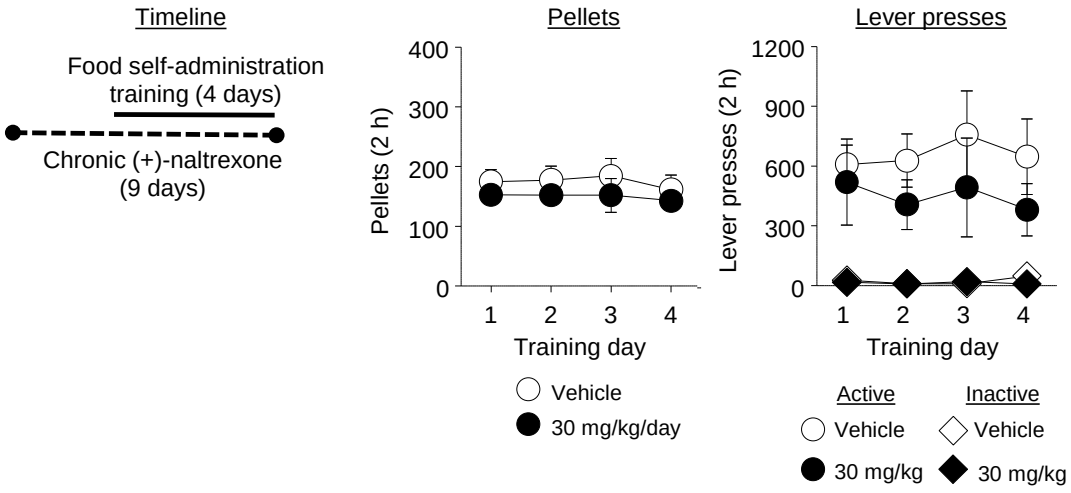

Figure S1. Chronic (minipump) delivery of (+)-naltrexone had no effect on palatable food self-administration. Data are mean ± SEM number of 45-mg food pellets, and active and inactive lever-presses during the 2-h daily self-administration sessions. Five days prior to training, rats were implanted subcutaneously with Alzet osmotic minipumps that delivered either vehicle (sterile water, n = 6) or (+)-naltrexone (30 mg/kg/day, n = 6) during the training period.

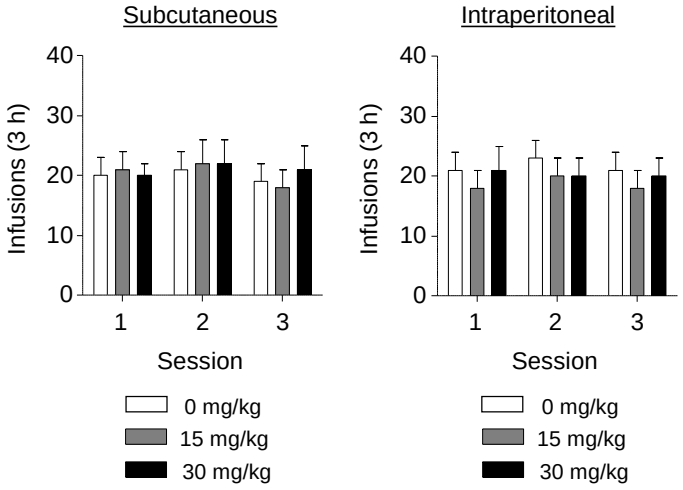

Figure S2. Acute systemic injections of (+)-naloxone had no effect on ongoing heroin self-administration. Data are mean ± SEM of heroin infusions (0.1 mg/kg/infusion) during the first, second, and third 3-h daily sessions. Systemic injections (subcutaneous or intraperitoneal) of (+)-naloxone (0, 15, and 30 mg/kg; n = 11) were given 10-15 min prior to the start of the first 3-h daily session.

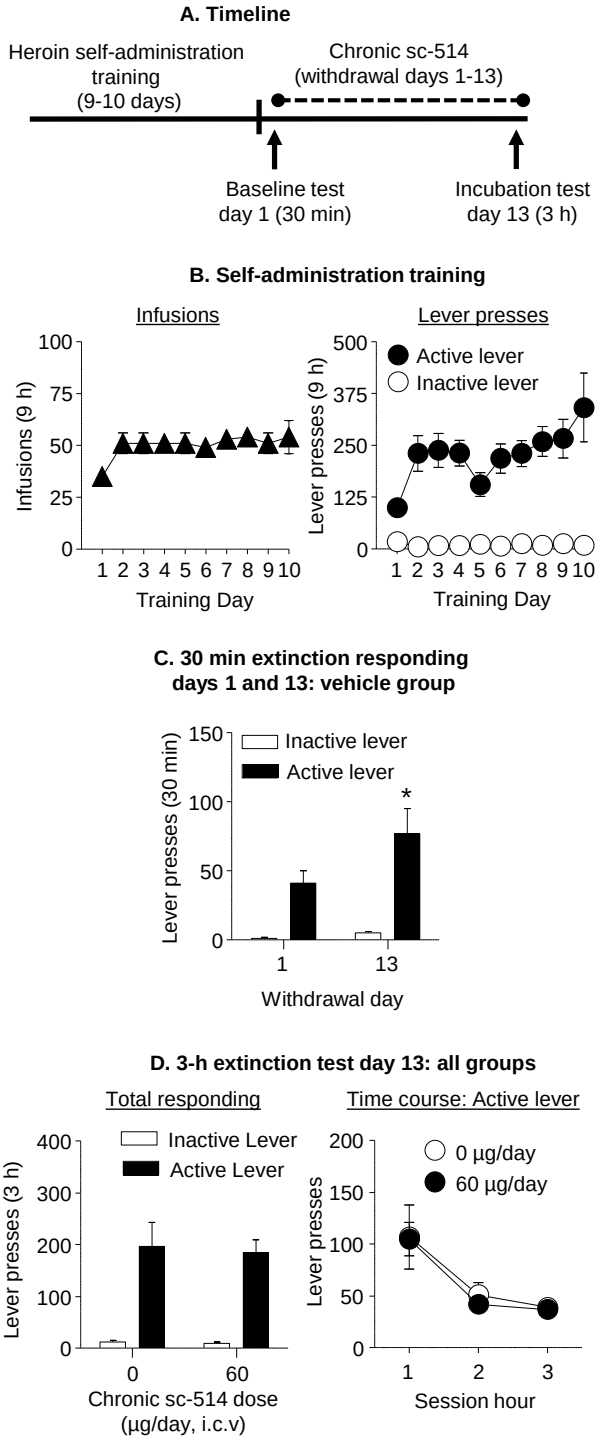

Figure S3. Chronic intracerebroventricular (i.c.v.) delivery of the NFkB antagonist SC-514 during the withdrawal phase had no effect on cue-induced heroin seeking on withdrawal day 13. (**A**) Timeline of the experiment. *Heroin self-administration training.* Data are mean ± SEM number of heroin infusions (0.1 mg/kg/infusion), and active and inactive lever presses during the nine 9-h daily self-administration sessions (total *n* = 28). During training, active lever presses were reinforced on an FR1 20-sec timeout reinforcement schedule and heroin infusions were

paired with a 5-sec tone-light cue. (**D**) *Extinction test withdrawal day 1 and 13 (vehicle group):* Data are mean ± SEM of active and inactive lever presses in the vehicle-treated group (n = 14) during the 30-min extinction test on withdrawal day 1 and the first 30 min of the 3-h extinction test on withdrawal day 13. *Different from withdrawal day 1, p = 0.045. (**D**) *Extinction test withdrawal day 13:* Data are mean ± SEM responses on the previously active lever and on the inactive lever during the 3-h extinction test in rats implanted (subcutaneous) on withdrawal day 1 with osmotic minipumps (Alzet model 2002) that delivered (via PE-50 tubing connected to a 30-gauge injector inserted into a 23-gauge guide cannula (Plastics One) (7)) either vehicle (50% DMSO/50% sterile water, 12 µl/day, n = 14) or sc-514 (60 µg/day, 12 µl/day, n = 14) into the lateral ventricle (unilateral). During testing, lever presses led to contingent presentations of the tone-light cue previously paired with heroin infusions during training, but not heroin. The rats were implanted with a 23-gauge guide cannula 2 mm above the lateral ventricle just prior to the intravenous catheter surgery, using the stereotaxic coordinates (17) of AP −0.9 mm, ML ±1.5 mm, and DV -2.0 mm; the coordinates were measured from Bregma (nosebar at -3.3 mm) and were based on our previous study (18). The sc-514 ((Enzo Life Sciences) dose for chronic lateral ventricle delivery was based on a previous paper of Koo *et al.* (19).

Supplemental References

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