Supporting Information

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SI Materials and Methods: Quantification of Heroin Metabolites

Sample Collection. Trunk blood was collected in a 1:1 ratio with acetate buffer (0.1 M sodium acetate/0.1 M acetic acid/50 g/L NaF, pH 6.0), and serum was then separated by centrifugation at 10,621 × *g* for 5 min. Rat brains were dissected following decapitation, and were homogenized in the acetate buffer at a ratio of 6 mL per brain. An internal standard solution [100 μL of 50 ng/mL each of deuterated heroin, deuterated morphine, and deuterated 6-acetylmorphine (6-AM)] was added to 100 μL of serum or brain homogenate, followed by protein precipitation with 400 μL of ice-cold acetonitrile/methanol (85:15). The samples were immediately vortexed for 30 s, placed in a −20 °C freezer for 10–20 min, and then centrifuged at 2,151 × *g* for 10 min. The supernatant was transferred to a new microcentrifuge tube and then dried at ambient temperature in a GeneVac EX-2 Evaporation System (GeneVac). Dried samples were resuspended in 100 μL of acetonitrile, vortexed, centrifuged at 10,000 rpm for 5 min, and then transferred to LC vials. The samples were stored at 4 °C until the liquid chromatography-tandem mass spectrometry (LC-MSMS) analysis.

Preparation of Standards. All of the standard solutions were prepared in acetonitrile and stored at 4 °C until analysis. A single internal standard stock solution was prepared for all of the compounds, with a final concentration of 50 ng/mL for each deuterated standard. Calibration standards were prepared at concentrations that ranged from 50 pg/mL to 100 ng/mL, with an internal standard concentration of 50 ng/mL.

LC-MSMS Analysis. LC-MSMS experiments were performed using an Agilent 6490 Triple Quadrupole LC/MS with Jet Stream and iFunnel technology (Agilent Technologies), electrospray ionization in positive mode (ESI+), and multiple reaction monitoring (MRM). Chromatographic separation was achieved on a Poroshell 120 EC-C8 column (2.1 mm × 50 mm, 2.7 μm; Agilent Technologies) at a flow rate of 350 μL/min, with a sample injection of 1 μL. The total run time for each sample analysis was 20 min and consisted of an elution gradient that began with a 2-min hold at 5% B, ramping to 95% B over 8 min, and holding for 5 min, with a final reequilibration time of 5 min. The mobile phase A consisted of water with 0.1% formic acid, pH 7, and mobile phase B was acetonitrile with 0.1% formic acid. To decrease the possibility of sample carryover, two blank injections were run between the sample injections.

The MS conditions consisted of a source temperature of 200 °C with drying gas flow of 12 L/min, capillary voltage of 3,500 V, and a nebulizer pressure of 20 psi. A sheath gas flow of 12 L/min and a temperature of 400 °C were used for the Agilent Jet Stream technology. MRM conditions included a dwell time and fragmentor voltage of 200 ms and 380 V, respectively. All of the other MRM parameters are listed in Table S2.

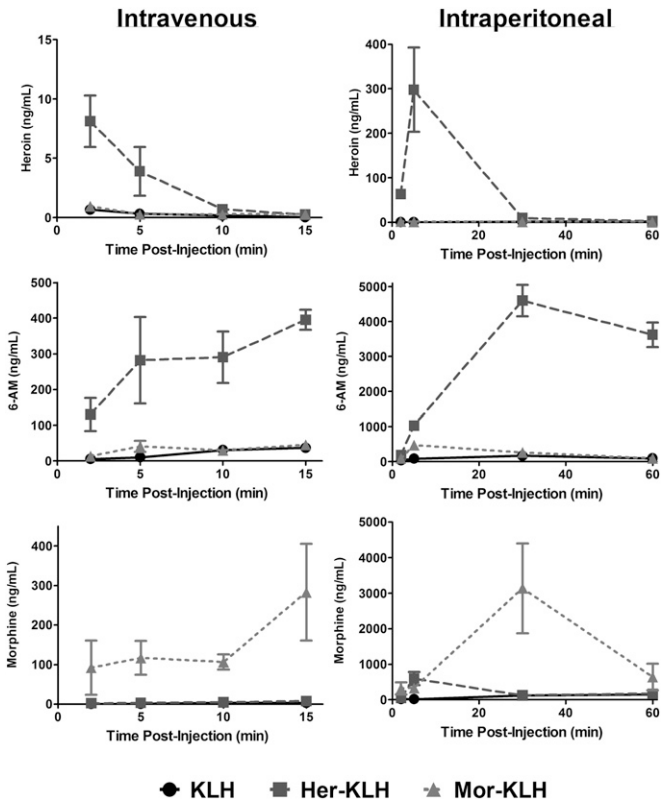

Fig. S1. Timelines of blood serum concentrations of the brain-penetrant metabolites. Blood levels of heroin (*Top*), 6-acetylmorphine (*Middle*), and morphine (*Bottom*) were measured in rats injected with either 0.5 mg/kg into the tail vein (*Left*) or 1.5 mg/kg intraperitoneally (*Right*). Heroin–KLH (keyhole limpet hemocyanin) vaccination promoted sequestration of heroin and 6-acetylmorphine in blood, whereas Morphine–KLH vaccination sequestered morphine. $n = 5–6$ per time point. The data are expressed as mean ± SEM.

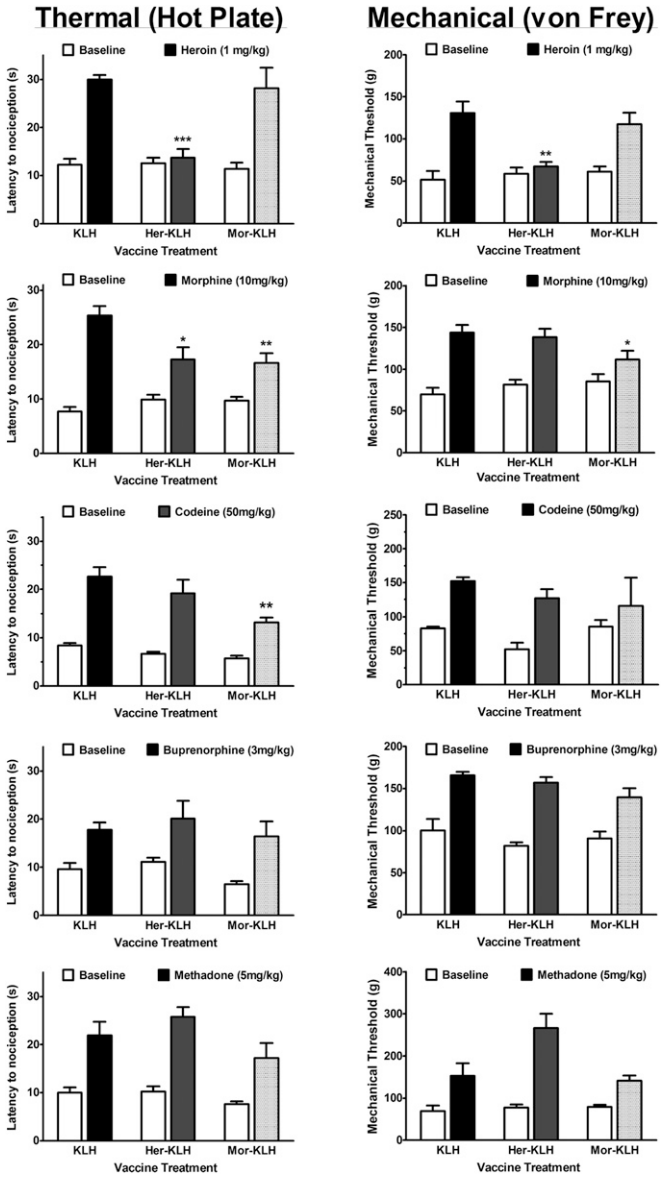

Fig. S2. Individual opiate antinociceptive effects compared with baseline. Latencies to nociceptive behavior on a 54 °C hot plate (*Left*) and threshold force exerted by von Frey filaments to produce paw withdrawal (*Right*) are shown. Comparisons with baseline are shown for KLH-, Heroin–KLH-, and morphine–KLH-vaccinated rats. The opioid drugs tested included (top to bottom): 1 mg/kg heroin, 10 mg/kg morphine, 50 mg/kg codeine, 3 mg/kg buprenorphine, and 5 mg/kg methadone. $n = 6–7$ per group. The data are expressed as mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with KLH controls.

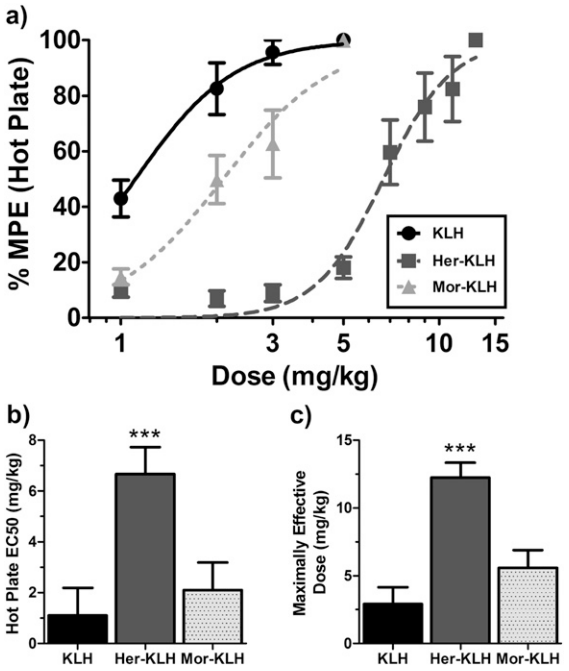

Fig. S3. Cumulative dose–response of heroin in hot plate antinociception. Drug-naïve vaccinated rats were injected with incrementally increasing heroin (s.c.) every 20 min and evaluated for latency for thermal nociception. (*A*) Dose–response curves of percent maximum possible effect. Her–KLH vaccinated rats show significant increases in the 50% effective concentration (EC_{50}; *B*), as well as the dose required to obtain maximal antinociception (*C*). $n = 8–9$ per group. Data are expressed as mean ± SEM ***$P < 0.001$, compared with KLH controls.

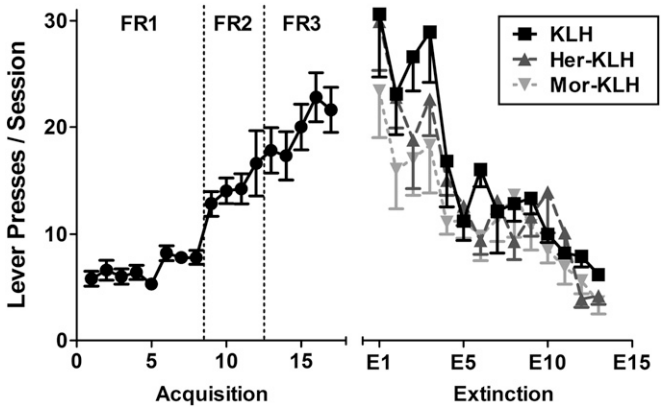

Fig. S4. Acquisition of heroin self-administration and extinction curves for each vaccination group before reinstatement testing. Rats were trained to press under a fixed-ratio 3 (FR3) schedule of reinforcement (i.e., three lever press to receive a dose of drug) until stable responding was achieved, and then cues. Next, rats underwent extinction session where cues and heroin infusions were not presented upon lever presses. The vaccine groups were split equally based on the final two acquisition sessions. The vaccination process did not significantly alter the rate or magnitude of extinction. $n = 9$ per group. The data are expressed as mean ± SEM.

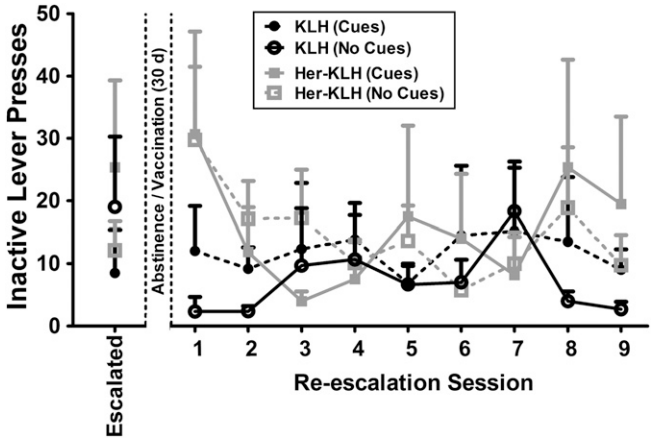

Fig. S5. Inactive lever responses during reescalation of self-administration. Rats typically pressed fewer than 20 inactive lever presses during the 12 h heroin extended access sessions, with occasional spikes based on increased activity of single subjects (accompanied by time-out active lever presses and food pellet responses). No differences are observed across time or treatment. n = 6–8 per group. The data are expressed as mean + SEM.

Table S1. Average (± SEM) KLH titer response by experiment

Experimental group	Her–KLH titer	Mor–KLH titer
Metabolite distribution (i.p.)	102,000 ± 22,000	91,000 ± 13,000
Metabolite distribution (i.v.)	49,000 ± 11,000	89,000 ± 48,000
Opioid antinociception	77,000 ± 14,000	77,000 ± 10,000
Hot plate dose–response	181,000 ± 35,000	138,000 ± 32,000
Conditioned place preference	53,000 ± 17,000	77,000 ± 29,000
Reinstatement	61,000 ± 9,000	89,000 ± 11,000
Reescalation (with cues)	40,000 ± 13,000	N/A
Reescalation (without cues)	68,000 ± 23,000	N/A

Table S2. MRM parameters for LC-MSMS analysis

Substance	Retention time, min	MRM 1, *m/z*	Collision energy, V
Morphine	7.3	286.15 > 152.0	64
Morphine-d_3		289.12 > 165.1	44
6-AM	8.0	328.16 > 210.9	20
6-AM-d_3		331.18 > 165.1	44
Heroin	8.5	370.17 > 164.9	56
Heroin-d_9		379.23 > 164.9	72