A Model of Δ⁹-Tetrahydrocannabinol Self-administration and Reinstatement That Alters Synaptic Plasticity in Nucleus Accumbens

Supplemental Information

Supplemental Methods and Materials

Experimental Procedures

Subjects and surgery. Male Sprague–Dawley rats (250–300 g, Charles River Laboratories) were maintained on a 12–12 hr reverse light-dark cycle with *ad-libitum* food and water prior to operant training. After 1 week of vivarium acclimation, rats were implanted with indwelling jugular catheters. Rats were surgically implanted with intravenous silastic catheters in the right jugular vein under anesthesia with ketamine (87.5 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.). Ketorolac (3 mg/kg, i.p.) was administered prior to surgery and as needed postoperatively to provide analgesia. Prophylactic antibiotic (Cefazolin 10 mg/0.1 ml, i.v.) was administered during surgery. The catheter was secured to the vein with silk sutures and was passed subcutaneously to the middle of the back where it terminated in a connector consisting of a modified 22-gauge cannula (Plastics One, Roanoke, VA) embedded in dental cement attached to surgical mesh (Atrium, Hudson, NH). Catheters were flushed daily with heparin (0.1 mL of 100 IU) until the end of self-administration, and catheter patency was confirmed at the end of each study. Food was restricted to 25 g standard chow the day prior to food training. All experiments were performed in the dark cycle. Experimental procedures were approved by the Animal Care and Use Committee of the Medical University of South Carolina and performed in accordance with National Institutes of Health guidelines.

Body temperature and THC blood measurements. Body temperatures were recorded with a rectal probe (CWE, Inc) both before vaporization and immediately after involuntary vapor exposure to determine uptake of the drug. Similarly, body temperature measurements were made before and after a self-administration session. Blood samples (500 µl) were collected by puncturing the lateral tail vein with a heparin-coated 23 G butterfly needle approximately 1 hr after vapor exposure or self-administration and left at 4°C for 24 h. The samples were centrifuged at 3,000 x g for 10 min at 4°C to collect serum. The samples were stored in a -80°C freezer until use. Levels of THC and its metabolites were determined using an ELISA kit (Bioo Scientific) according to the manufacturer's instructions. This ELISA kit also detects the major metabolites of THC including 11-Hyrdoxy-Delta9-THC and 11-Nor-9-Carboxy-Delta9-THC.

Locomotor Activity

Rats were first acclimated to the locomotor activity chambers for 3 one-hour sessions to eliminate any response to novelty. On the fourth session, rats were injected with rimonabant (3 or 10 mg/kg, ip) or vehicle 30 minutes prior to being placed in the locomotor activity chamber. A photocell apparatus (AccuScan Instruments) was used to record movement using software that estimated distance traveled based on consecutive breaking of adjacent photobeams for 90 min to assess effects of rimonabant on habituated locomotor activity akin to the conditions experienced during operant self-administration. A within-subject randomized crossover design with a 3-day inter-trial interval was used to compare rimonabant and vehicle.

Electrophysiology

Slice preparation. Rats were anesthetized with ketamine (100 mg/kg), decapitated, and

coronal brain slices (250 μm) made using a vibratome (VT1200S, Leica). Cutting was performed in ice cold ACSF at 4°C (in mM: 126 NaCl, 1.4 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 2.5 KCl, 2.0 sodium pyruvate, 0.4 ascorbic acid, 5 kynurenic acid, 0.05 D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5); bubbled with 95% O_2 and 5% CO_2). After cutting, slices were stored for ≥45 min at 25°C.

AMPA/NMDA ratio. Recordings started no earlier than 10 min after the cell membrane was ruptured, to allow diffusion of the internal solution into the cell. AMPA currents were first measured at −80 mV to ensure stability of response. Then the membrane potential was gradually increased until +40 mV. Recording of currents was resumed 5 min after reaching +40 mV to allow stabilization of cell parameters. Currents composed of both AMPA and NMDA components were then obtained. Then D-AP5 was bath- applied (50 μM) to block NMDA currents, and recording of AMPA currents at +40 mV was started after 2 min. NMDA currents were obtained by subtracting the AMPA currents from the total current at +40 mV.

sEPSC recordings. Spontaneous EPSCs (sEPSC) were recorded in the whole cell voltage-clamp at -80 mV. sEPSCs were detected using a template generated from averaging typical synaptic events of each cell, using AxoGraph X software (AxoGraph Scientific). The template was slid along the data trace one point at a time. At each position, this template was optimally scaled and offset to fit the data. The detection criterion was set to 3.5 standard deviations of baseline noise.

Dendritic Spine Labeling and Quantification

Dendritic spine labeling and quantification procedures were based on Seabold et al. and were similar to those described previously with some modifications (1, 2). Rats were deeply anesthetized with ketamine HCl (87.5 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.)

before being decapitated. Brains were removed and 250 μm coronal sections containing NAcore were post-fixed for 30 min in 4% paraformaldehyde. Tungsten particles (1.3 μm diameter; Bio-Rad, Hercules, CA) were coated with the lipophilic carbocyanine dye DiI (Life Technologies, Grand Island, NY), and these DiI-coated particles were delivered diolistically into the tissue at 80 PSI using a Helios Gene Gun system (Bio-Rad) fitted with a polycarbonate filter with a 3.0 μm pore size (BD Biosciences, San Jose, CA). DiI was allowed to diffuse along neuronal axons and dendrites in PBS for 2 hours at room temperature. A confocal microscope (Leica) was used to image DiI-labeled sections using the Helium/ Neon 543-nm laser line. Micrographs of DiI-labeled neurons and dendrites were acquired via optical sectioning by a 63× oil immersion objective (numerical aperture=1.4) with pixel size 0.01 μm at XY plane and 0.13-μm intervals along the z axis. Images were deconvolved by Autoquant (Media Cybernetics), and a 3D perspective was rendered by the Surpass module of the Imaris software (Bitplane, Concord, MA). Spines on dendrites beginning at >75 μm and ending at ≤200 μm distal to the soma and after the first branch point were quantified from NAcore MSNs. Seven to nine segments, one segment per neuron, (45-55 μm each) were analyzed per animal. Minimum spine head diameter was set at ≥0.143 μm to reflect the Nyquist frequency resolution limits of the microscope.

Statistics

Statistics were performed using Prism (GraphPad Software, La Jolla, CA). Self-administration data were analyzed by one- or two-way ANOVA as appropriate followed by Sidak's multiple comparisons. Two-way repeated measures ANOVA was used to analyze all reinstatement behavior. When only two groups were compared, statistical significance ($p < 0.05$) was determined by Student's *t* test. Electrophysiological data were analyzed as

individual cells from 3-5 animals per group using a one-way ANOVA with Dunnett's post-hoc for multiple comparisons. Significance was set at $p \leq 0.05$ and all data are presented as mean ± SEM.

Supplemental Data and Tables

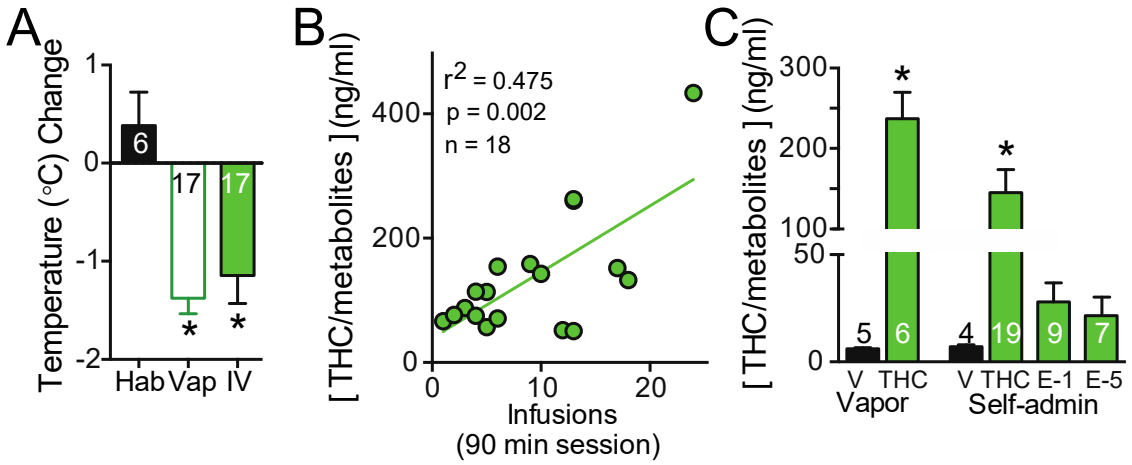

Figure S1. Validation of delivery of physiologically relevant THC levels. A) Hypothermia produced by vapor and intravenous THC+CBD (2.0+0.2 µg/infusion). Hab= habituation (no drug), Vap= at the end of vapor exposure, IV= at the end of a self-administration session (2.0+0.2 µg/infusion). *p<0.05, comparing pre- to post-THC+CBD exposure using a paired Student's t-test. **B)** Amount of THC and metabolites detected in serum correlates with number of infusions achieved during a THC+CBD self-administration session. **C)** Measurement of THC and metabolites in the serum of rats following THC+CBD vapor, intravenous THC+CBD self-administration, or after one (E-1) or five (E-5) days of extinction training.

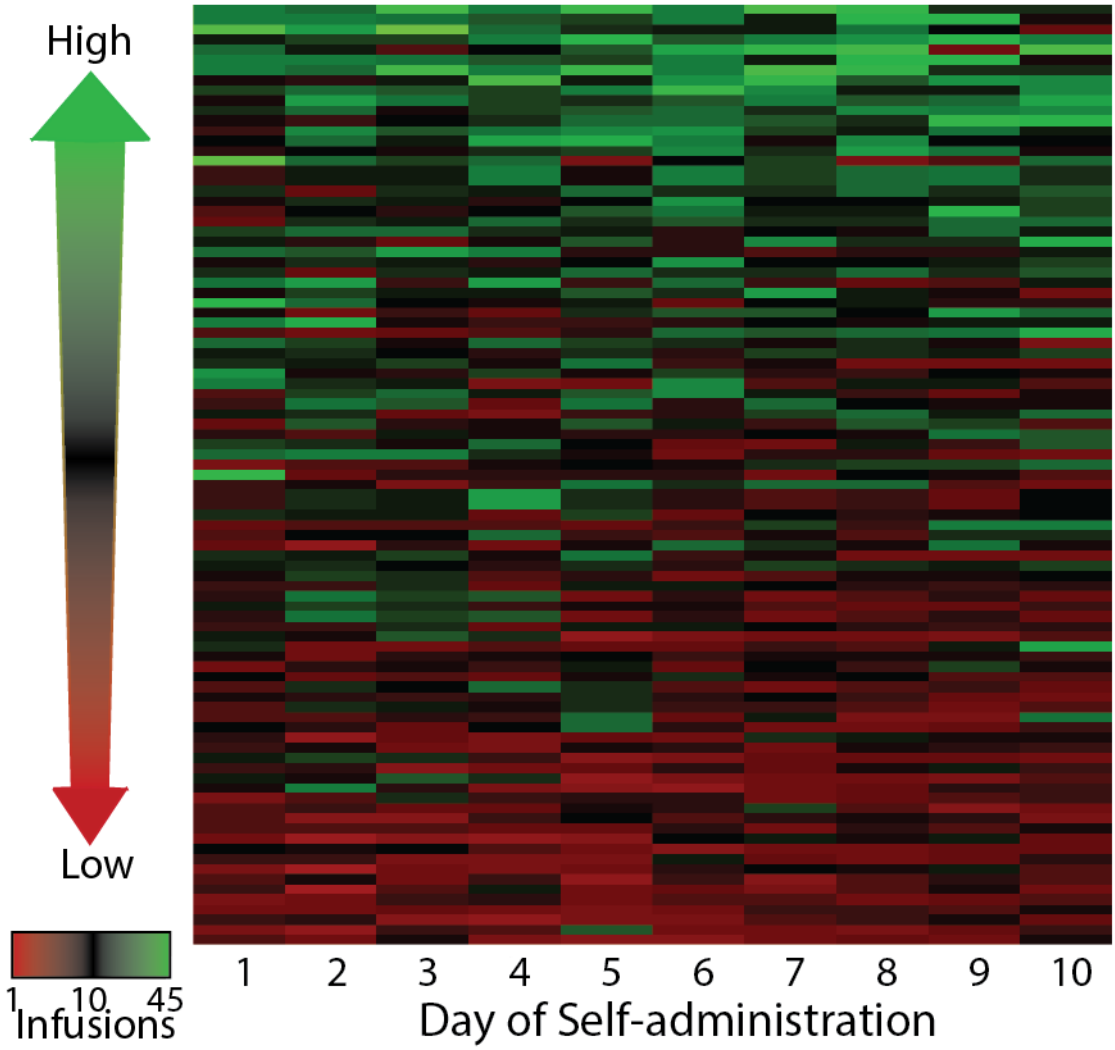

Figure S2. Individual differences in drug intake. A heat map showing individual variability in THC+CBD infusions throughout the 10 days of self-administration. Higher numbers of infusions are shown in green (max=45) and lower numbers of infusions are red (min=1). Note that 3 potential subgroups emerged based on drug intake with high users generally consuming >10 infusions per day throughout training and low users consuming <10. A subset of rats displayed a highly variable infusion rates between days.

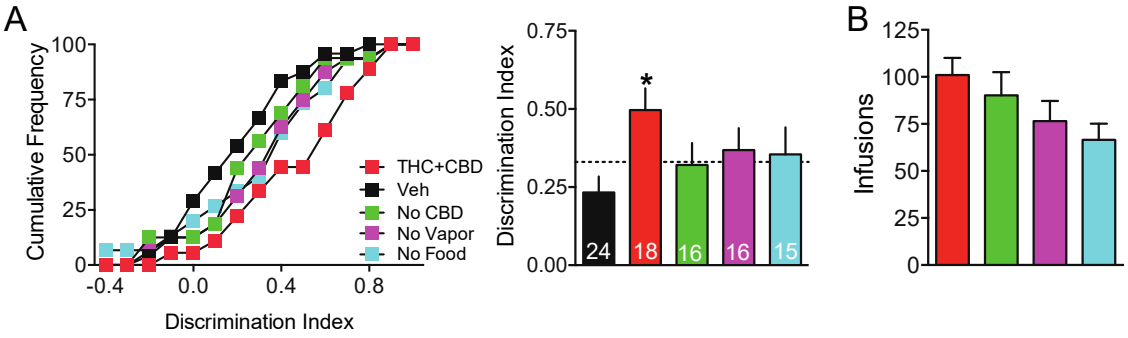

Figure S3. The treatment protocol in Figure 1A results in a higher discrimination index than when CBD, vapor pretreatment or food training was eliminated. A) Left panel illustrates a frequency plot of the discrimination index showing that THC+CBD is shifted to the right relative to all other treatments, indicating greater reinforcing value of this combination. Right panel compares the mean lever preference ratio, verifying the higher lever preference ratio for THC+CBD relative to Vehicle, with the other combinations having intermediate values. The dotted line indicates a 2:1 ratio of active to inactive lever pressing. N shown in the bar. *p< 0.05 comparing all treatments to vehicle. One-way ANOVA followed by a Dunnett's multiple comparisons test. **B)** Comparison of total infusion number between different treatment groups. There was a trend for THC+CBD combination rats to show the highest drug intake (one way ANOVA $F_{(3,61)}$=2.17, p=0.101).

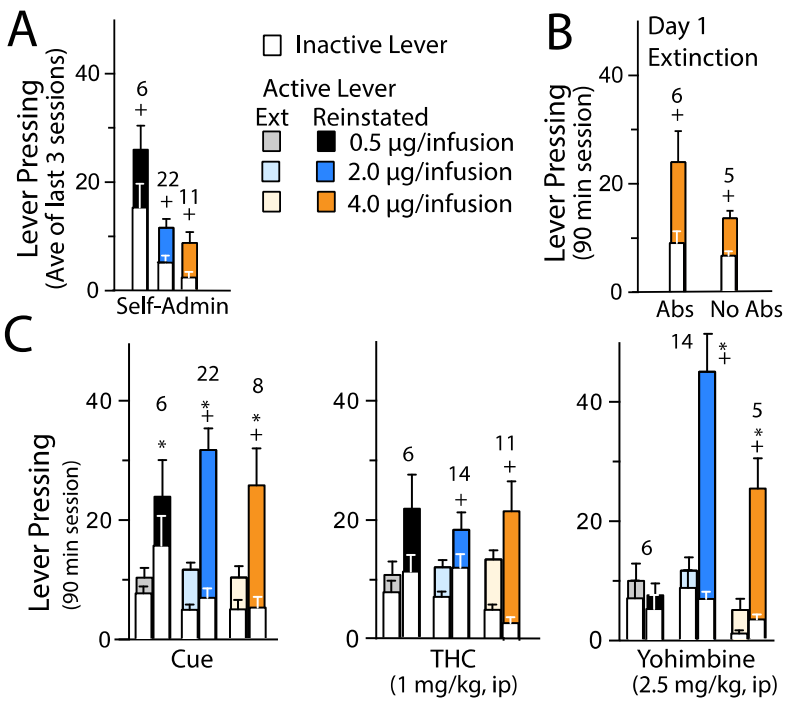

Figure S4. Reinstatement in rats extinguished from varying doses of THC self-administration. A) Comparison of lever pressing across doses of THC+CBD during the last three days of self-administration. Doses shown are for THC (CBD was co-administered at 10% the dose of THC). +p<0.05, comparing active and inactive lever pressing, using a paired Student's t-test. **B)** Comparison of day 1 extinction lever pressing between rats placed in 7-10 days of abstinence (Abs) versus rats placed into extinction training the day after discontinuing self-administration (No Abs) for the 4 µg/kg/infusion dose. +p<0.05, comparing active and inactive lever pressing. **C)** Comparison of lever pressing across doses after cue-induced, THC-primed (1 mg/kg, i.p.), or yohimbine-primed (2.5 mg/kg, i.p.) drug seeking. Lighter colored bars refer to the average of the last two extinction (Ext) sessions just prior to each reinstatement. *p<0.05, comparing active lever pressing between reinstatement and extinction within each dose and reinstatement modality using a 2-way ANOVA with repeated measures over lever and extinction vs. reinstatement, followed by a Sidak post hoc test. +p<0.05, comparing active and inactive lever pressing. In all panels N is shown over the bars. (See Table S3 for complete statistics).

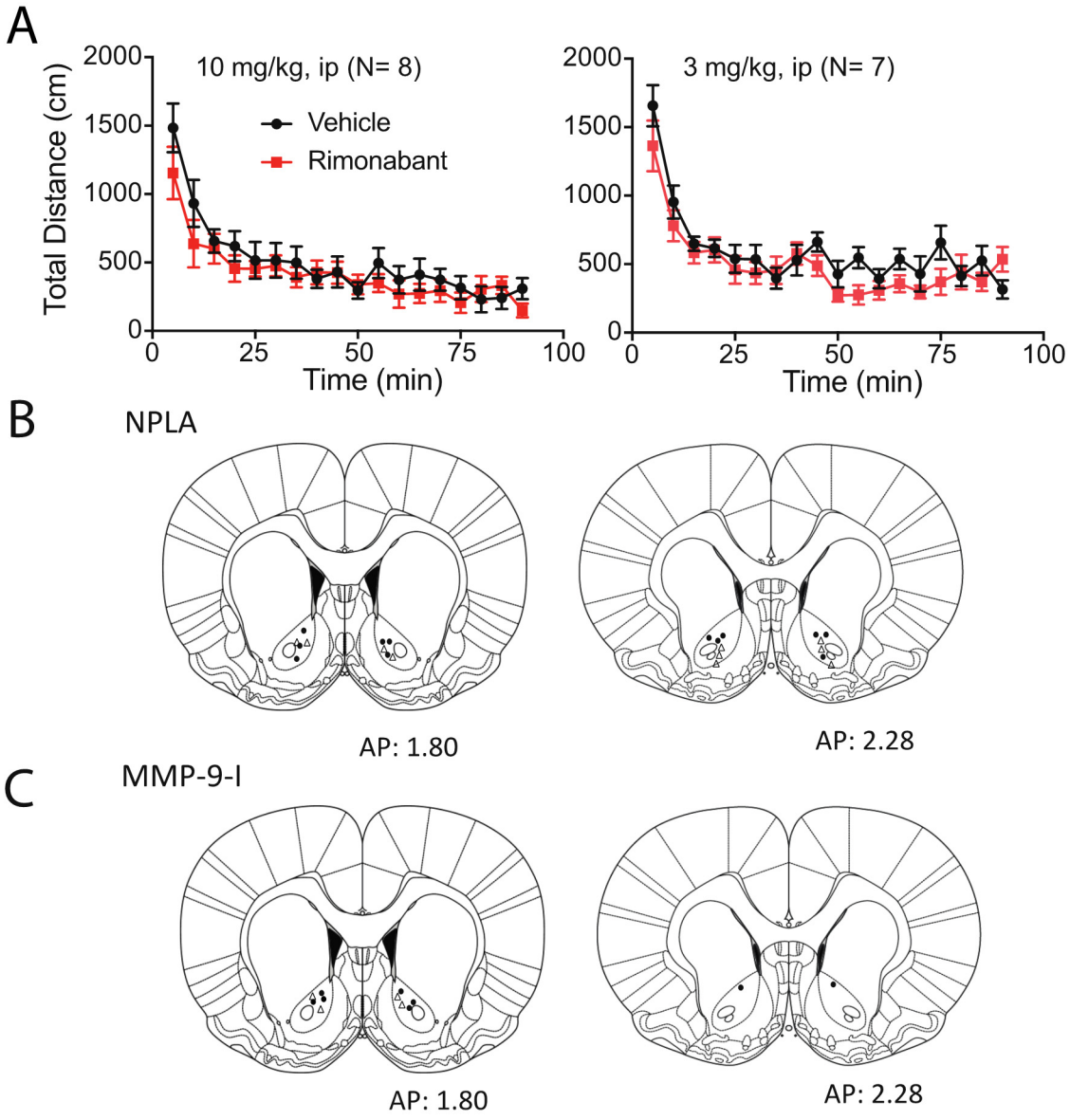

Figure S5. Lack of effect by rimonabant on locomotor activity and histological verification of inhibitor microinjections. A) Lack of effect by 10 or 3 mg/kg, ip of rimonabant on locomotor activity in an adapted open field. Rats were pretreated with rimonabant or vehicle in a crossover design using a 3 day inter-trial interval. Separate two-way ANOVA with repeated measures over time and treatment reveal an effect of time (10 mg/kg- $F_{(17,119)}=18.87$, $p<0.001$; 3 mg/kg- $F_{(17,102)}=19.95$, $p<0.001$), but no effect of treatment or interaction. **B)** Histologically determined location of microinjections of NPLA localized to the core subcompartment of the nucleus accumbens. Rats microinjected with

NPLA are indicated by closed circles and rats microinjected with vehicle are indicated by open triangles. **C)** Histologically determined location of microinjections of MMP-9-I localized to the core subcompartment of the nucleus accumbens. Rats microinjected with MMP-9-I are indicated by closed circles and rats microinjected with vehicle are indicated by open triangles.

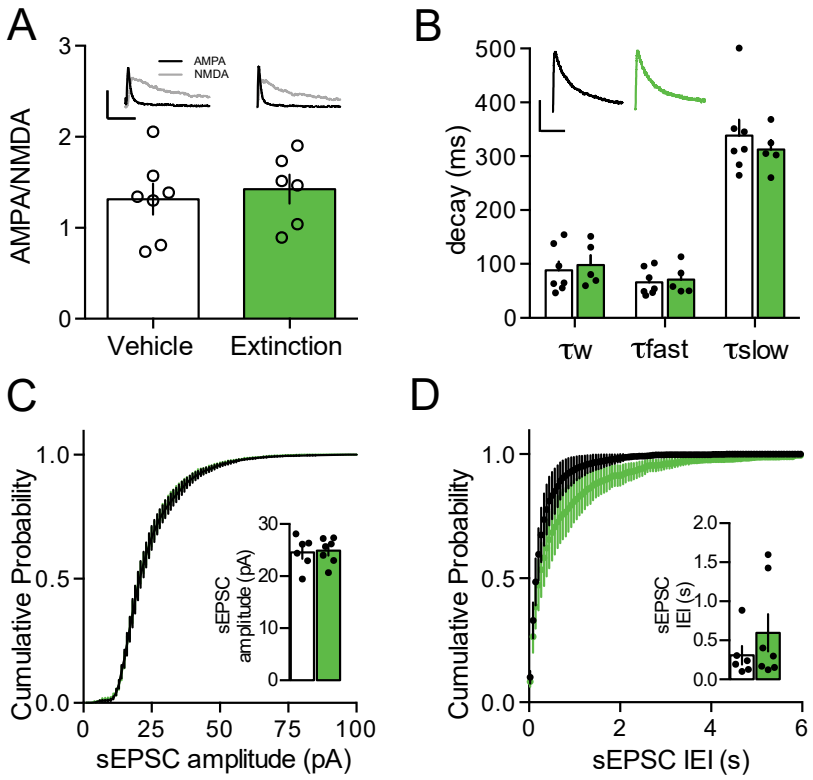

Figure S6. Extinction from THC+CBD self-administration does not change AMPA receptor signaling. A) Sample AMPA and NMDA current traces and averages showing that THC+CBD self-administration did not change AMPA/NMDA ratio. Calibration bars represent 100 pA and 10 ms. **B)** Sample traces of pharmacologically isolated NMDA currents and averages showing THC+CBD self-administration did not change the decay times of NMDA currents. Calibration bars represent 100 pA and 10 ms. **C)** Cumulative probability and mean values of amplitude for sEPSCs recorded from both treatment groups. **D)** Cumulative probability and mean values of Inter-Event-Intervals (IEI) for sEPSCs recorded from both treatment groups.

Table S1. Statistics for Figure 1B and D.

Day one and day ten of extinction- Comparing abstinent with nonabstinent (i.e. extinction conducted 24 hr after the last self-administration) lever pressing using a 2-way ANOVA with repeated measures over lever.

Day 1 extinction- 1.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	109.1	1	109.1	F (1, 99) = 1.312	P=0.2547
Abs or no Abs	2349	1	2349	F (1, 99) = 7.055	P=0.0092
Lever	4267	1	4267	F (1, 99) = 51.36	P<0.0001
Subjects (matching)	32956	99	332.9	F (99, 99) = 4.006	P<0.0001
Residual	8226	99	83.09		

Day 10 extinction- 1.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	269.0	1	269.0	F (1, 99) = 6.765	P=0.0107
Abs or no Abs	235.0	1	235.0	F (1, 99) = 2.013	P=0.1591
Lever	48.71	1	48.71	F (1, 99) = 1.225	P=0.2711
Subjects (matching)	11561	99	116.9	F (99, 99) = 2.937	P<0.0001
Residual	3936	99	39.76		

Table S2. Statistics for Figure 2.

Figure 2: Reinstatement to cue, THC or THC+CBD priming injection or yohimbine. Comparing reinstatement levels of active and inactive lever pressing between the average of the last two days of extinction just prior to reinstating to reinstatement pressing, using a two-way ANOVA with repeated measures over both active versus inactive lever and extinction versus reinstatement.

Statistics for Figure 2A

Cue – 1.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2987	1	2987	F (1, 24) = 28.02	P<0.0001
Ext v Cue	3715	1	3715	F (1, 24) = 18.94	P=0.0002
Lever	3108	1	3108	F (1, 24) = 19.95	P=0.0002
Subjects	10878	24	453.2		
Residual	2559	24	106.6		

Yoh – 1.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3281	1	3281	F (1, 7) = 11.32	P=0.0120
Ext v Yoh	5913	1	5913	F (1, 7) = 14.66	P=0.0065
Lever	4560	1	4560	F (1, 7) = 17.54	P=0.0041
Subjects	8042	7	1149		
Residual	2028	7	289.7		

THC prime – 1.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	41.34	1	41.34	F (1, 5) = 2.171	P=0.2006
Ext v Prime	36.26	1	36.26	F (1, 5) = 0.535	P=0.4973
Lever	1021	1	1021	F (1, 5) = 9.152	P=0.0292
Subjects	543.8	5	108.8		
Residual	95.22	5	19.04		

Statistics for Figure 2B

Cue – Vehicle

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	64.51	1	64.51	F (1, 6) = 2.94	P=0.1373
Ext v Cue	182.6	1	182.6	F (1, 6) = 5.95	P=0.0510
Lever	261.1	1	261.1	F (1, 6) = 15.7	P=0.0074
Subjects	508.8	6	84.74		
Residual	131.7	6	21.95		

Yoh – Vehicle

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	152.5	1	152.5	F (1, 5) = 1.102	P=0.3420
Ext v Yoh	1343	1	1343	F (1, 5) = 4.793	P=0.0802
Lever	137.8	1	137.8	F (1, 5) = 0.722	P=0.4343
Subjects	2406	5	481.3		
Residual	692.2	5	138.4		

THC prime – Vehicle

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.080	1	0.080	F (1, 6) = 0.005	P=0.9433
Ext v Prime	203.6	1	203.6	F (1, 6) = 6.834	P=0.0399
Lever	19.72	1	19.72	F (1, 6) = 0.599	P=0.4684
Subjects	383.7	6	63.96		
Residual	35.83	6	14.62		

Statistics for Figure 2C

Vehicle prime – 1.0 µg/infusion THC+CBD

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	7.656	1	7.656	F (1, 9) = 1.175	P=0.3066
Ext v Prime	8.556	1	8.556	F (1, 9) = 0.281	P=0.6092
Lever	97.66	1	97.66	F (1, 9) = 2.666	P=0.1369
Subjects	1783	9	198.1		
Residual	58.66	9	6.517		

THC+CBD prime – 1.0 µg/infusion THC+CBD

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	12.66	1	12.66	F (1, 9) = 1.265	P=0.2898
Ext v Prime	20.31	1	20.31	F (1, 9) = 0.524	P=0.4877
Lever	31.51	1	31.51	F (1, 9) = 0.747	P=0.4099
Subjects	2600	9	288.9		
Residual	90.03	9	10.00		

Table S3. Statistics for Figure S4.

Figure S4A: Self-administration (average of the last 3 days)- Comparing active with inactive lever using a two-tailed paired Student's t-test

Dose	N	df	t	probability
O.5 µg/infusion	6	5	2.83	0.037
2.0 µg/infusion	22	21	6.80	<0.001
4.0 µg/infusion	11	10	3.99	0.003

Figure S4B: Day one of extinction- Comparing abstinent with nonabstinent (i.e. extinction conducted 24 hr after the last self-administration) lever pressing at each dose using a 2-way ANOVA with repeated measures over lever.

4.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.456	1	1.456	F (1, 9) = 0.021	P=0.8873
Abs no Abs	48.55	1	48.55	F (1, 9) = 0.276	P=0.6116
Lever	1118	1	1118	F (1, 9) = 16.31	P=0.0029
Subjects (matching)	1579	9	175.4	F (9, 9) = 2.560	P=0.0888
Residual	616.8	9	68.54		

Figure S4C: Reinstatement to cue, THC priming injection or yohimbine. Comparing reinstatement levels of active and inactive lever pressing between the average of the last two days of extinction just prior to reinstating to reinstatement pressing, using a two-way ANOVA with repeated measures over both active versus inactive lever and extinction versus reinstatement.

Cue – 0.5 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	46.76	1	46.76	F (1, 5) = 7.440	P=0.0414
Ext v Cue	765.0	1	765.0	F (1, 5) = 3.832	P=0.1076
Lever	173.3	1	173.3	F (1, 5) = 4.565	P=0.0857
Subjects	950.9	5	190.2		
Residual	31.43	5	6.285		

Cue – 2.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1454	1	1454	F (1, 20) = 16.06	P=0.0007
Ext v Cue	2006	1	2006	F (1, 20) = 24.68	P<0.0001
Lever	5038	1	5038	F (1, 20) = 43.39	P<0.0001
Subjects	2828	20	141.4		
Residual	1811	20	90.53		

Cue – 4.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	520	1	520	F (1, 7) = 7.040	P=0.0328
Ext v Cue	496.1	1	496.1	F (1, 7) = 8.124	P=0.0247
Lever	1391	1	1391	F (1, 7) = 8.525	P=0.0223

Subjects	982.0	7	140.3		
Residual	517.1	7	73.87		

THC prime – 0.5 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	88.17	1	88.17	F (1, 5) = 5.747	P=0.0618
Ext v Prime	280.2	1	280.2	F (1, 5) = 2.452	P=0.1781
Lever	266.7	1	266.7	F (1, 5) = 5.235	P=0.0708
Subjects	993.2	5	198.6		
Residual	76.71	5	15.34		

THC prime – 2.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	5.558	1	5.558	F (1, 12) = 0.299	P=0.5946
Ext v Prime	387.8	1	387.8	F (1, 12) = 4.241	P=0.0618
Lever	382.3	1	382.3	F (1, 12) = 8.086	P=0.0148
Subjects	1507	12	125.6		
Residual	223.2	12	18.60		

THC prime – 4.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	192.4	1	192.4	F (1, 10) = 2.453	P=0.1484
Ext v Prime	76.45	1	76.45	F (1, 10) = 0.753	P=0.4060
Lever	2415	1	2415	F (1, 10) = 33.19	P=0.0002
Subjects	1510	10	151.0		
Residual	784.1	10	78.41		

Yoh – 0.5 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.260	1	0.260	F (1, 5) = 0.043	P=0.8424
Ext v Yoh	27.09	1	27.09	F (1, 5) = 0.889	P=0.3890
Lever	58.59	1	58.59	F (1, 5) = 1.539	P=0.2698
Subjects	576.2	5	115.2		
Residual	29.68	5	5.935		

Yoh – 2.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3860	1	3860	F (1, 12) = 27.08	P=0.0002
Ext v Yoh	2762	1	2762	F (1, 12) = 17.45	P=0.0013
Lever	5775	1	5775	F (1, 12) = 60.52	P<0.0001
Subjects	1948	12	162.3		
Residual	1711	12	142.5		

Yoh – 4.0 µg/infusion

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	423.3	1	423.3	F (1, 4) = 10.68	P=0.0309
Ext v Yoh	460.8	1	460.8	F (1, 4) = 9.691	P=0.0358
Lever	980.0	1	980.0	F (1, 4) = 26.35	P=0.0068
Subjects	220.2	4	55.05		
Residual	158.6	4	39.64		

Supplemental References

1. Seabold GK, Daunais JB, Rau A, Grant KA, Alvarez VA (2010): DiOLISTIC Labeling of Neurons from Rodent and Non-human Primate Brain Slices.e2081.
2. Stefanik MT, Kupchik YM, Kalivas PW (2016): Optogenetic inhibition of cortical afferents in the nucleus accumbens simultaneously prevents cue-induced transient synaptic potentiation and cocaine-seeking behavior. *Brain structure & function*. 221:1681-1689.