# Supplemental Information

## Supplemental Methods & Materials

*Intracranial self-stimulation (ICSS)*

Rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.; 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 stimulation current was adjusted (final range: 110 – 210 µA) for each rat to the lowest value that would sustain a reliable rate of responding (average of 40 responses per 50 s). After the minimal effective current was found for each rat, it was kept constant throughout the remainder of training and testing. These procedures have been described in detail (1, 2).

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)).

In order to test the effects of very low doses of salvinorin A (salvA) on reward thresholds, salvA was dissolved in a vehicle of Tween 80, ethanol, and saline; (1:1:8) and administered subcutaneously (s.c.), in order to replicate conditions described in (3).

A subset of the 59 rats used for ICSS (*N* = 14) were used to assess how long nor-binaltorphimine (norBNI) would block the reward-decreasing effects of salvA. Starting 22 days after treatment with norBNI (12 days after the cocaine challenge day), rats were challenged with either vehicle or salvA (2.0 mg/kg) once every 7-14 days for a total of 86 days after treatment with norBNI. Prior to each challenge treatment, baseline thresholds were determined as above.

*Western blotting*

After treatments and sacrifice by decapitation, brains were rapidly removed and frozen in isopentane kept on dry ice. Brains were then sliced on a cryostat (HM 505 E, Microm, Walldorf, Germany) kept at -20$^{o}$C until the rostral nucleus accumbens (NAc) and caudate putamen (CPu) were exposed (Bregma 2.20 mm). Bilateral tissue punches 1-mm in length were taken of the NAc (diameter: 1 mm) and CPu (diameter: 2 mm) and placed in Eppendorf tubes kept on dry ice. Tissue was sonicated (Sonic Dismembrator 60, Fisher Scientific, Pittsburgh, PA) in 100 µl 1% sodium dodecyl sulfate (SDS) to break apart cell membranes and nucleic acids. Protein content was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA), and the concentration of each sample was adjusted to 2.0 mg/ml protein. NuPAGE LDS (lithium dodecyl sulfate) sample buffer (Invitrogen, Carlsbad, CA) and 50 mM dithiothreitol were added to each sample prior to heating at 70$^{o}$C for 10 min. Twenty µg of each sample were then loaded onto NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen) for separation by gel electrophoresis. Proteins were subsequently transferred to polyvinylidene fluoride membrane (PVDF) (PerkinElmer Life Sciences, Boston, MA). Nonspecific binding sites on the membranes were blocked for 2 hr at room temperature in blocking buffer (5% nonfat dry milk in PBS and 0.1%

Tween 20 [PBS-T]). Blots were then incubated in primary antibody in PBS-T overnight at 4$^{o}$C. Blots were washed 4 x 15 min in PBS-T and then incubated in secondary antibody (1:5000 goat anti-rabbit [or anti-mouse] horseradish peroxidase-linked IgG [Vector Laboratories, Burlingame, CA]) for 2 hr at room temperature. Blots were washed 4 x 15 min in PBS-T, followed by immunological detection using Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). Antibodies were stripped from the blots by incubation with stripping buffer (62.5 mM Tris, 2% SDS, 100 mM b-mercaptoethanol, pH 6.8) for 15 min at 50$^{o}$C. Blots were subsequently re-blocked and probed with 1:20,000 anti-actin (Sigma, St. Louis, MO). SeeBlue Plus 2 (Invitrogen) pre-stained standards were run for molecular weight estimation. Primary antibodies used were: 1:4000 monoclonal anti-P-ERK 44/42, 1:4000 polyclonal anti-ERK2, 1:1000 monoclonal anti-P-CREB, and 1:1000 polyclonal CREB (Cell Signaling Technology, Beverly, MA). P-ERK antibody detects both 42 and 44 KDa ERK2 and ERK1 proteins, ERK2 is the 42 kDa isoform, P-CREB and CREB antibodies detect 42 kDa CREB proteins, and actin was detected at 42 kDa.

Protein immunoblots were analyzed using Kodak 1D Image Analysis software (Kodak, Rochester, NY). Relative optical densities were determined for each band of interest (ERK2, P-ERK 42 kDa, CREB, P-CREB, β-actin). To control for loading differences of protein, the optical density of each band was normalized with the corresponding optical density of β-actin. To allow for comparisons between blots, data were normalized to the vehicle-treated controls in each experiment. Data are expressed as the mean fold induction compared to vehicle control ± SEM

*Double label immunohistochemistry (IHC)*

Briefly, 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). Brains were postfixed for 3 d at 4$^{o}$C, then transferred to 20% glycerol in 50

mM phosphate buffer (PB; pH 7.4) at 4$^{o}$C until saturation (≥ 24 hr). Coronal sections (30 μM) were cut on a freezing microtome and free-floating sections were rinsed, treated with 2% hydrogen peroxide for 20 min, and then blocked for 2 hr at room temperature in AB media (0.3% Triton X-100 (TX), 2% normal horse serum (Invitrogen), and 1% bovine serum albumin (BSA, Sigma) in 0.01 M TBS). The sections were 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 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. Sections were incubated with avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories) for 30 min at room temperature and reacted with 0.05% 3,3'diaminobenzidine tetrahydrochloride containing nickel chloride in 0.01% hydrogen peroxide (DAB, Sigma) for 4 min. Rinsing in 0.01 M phosphate buffer terminated the reaction. Sections were subsequently rinsed and incubated for 30 min at RT in Avidin D and biotin to block avidin/biotin, and were then incubated in AB media (containing goat serum) for 2-hr to block nonspecific binding sites. Sections were incubated as above on a shaker overnight at room temperature with an antibody made in guinea pig directed against prodynorphin (AB5519, Chemicon, Billerica, MA), diluted 1:4,000 in AB media. The following day, sections were rinsed and incubated for 1 hr at room temperature in biotinylated goat anti-guinea pig immunoglobulin G secondary antibody (Vector Laboratories) diluted 1:200 in AB media. Sections were incubated with avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories) for 30 min at room temperature and then reacted with cacodylate-buffered 0.05% 3,3'diaminobenzidine tetrahydrochloride in 0.01% hydrogen peroxide (DAB, Sigma) for 1.5 min.

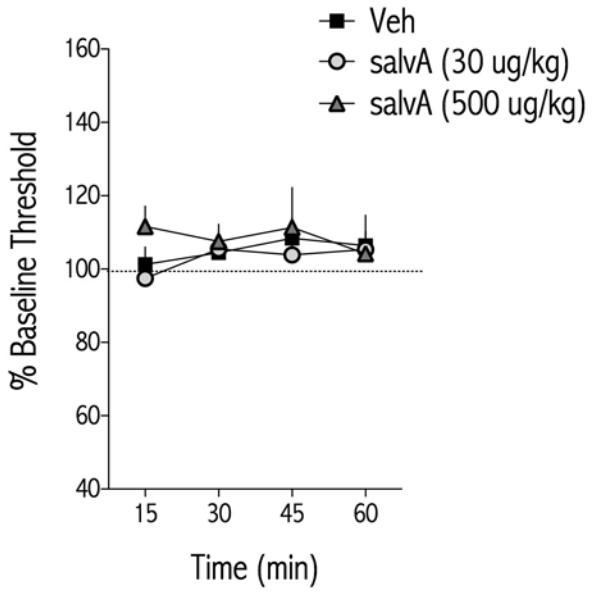


**Figure S1.** Low doses of salvA do not affect intracranial self-stimulation (ICSS) thresholds. Separate rats were treated acutely with vehicle (Tween 80; ethanol; saline; 1:1:8) or low doses of salvA (30 and 500 μg/kg, s.c.) and ICSS thresholds were measured immediately after injection for 60 min. salvA, salvinorin A; Veh, vehicle.

## Supplemental References

1. Carlezon WA, Jr., Chartoff EH (2007): Intracranial self-stimulation (ICSS) in rodents to study the neurobiology of motivation. *Nat Protoc*. 2:2987-2995.
2. Ebner SR, Roitman MF, Potter DN, Rachlin AB, Chartoff EH (2010): Depressive-like effects of the kappa opioid receptor agonist salvinorin A are associated with decreased phasic dopamine release in the nucleus accumbens. *Psychopharmacology (Berl)*. 210:241-252.
3. Carlezon WA, Jr., Bèguin C, Knoll AT, Cohen BM (2009): Kappa-opioid ligands in the study and treatment of mood disorders. *Pharmacol Ther*. 123:334-343.