Supporting Information

Chung et al. 10.1073/pnas.0811331106

SI Materials and Methods

Animals. Melanin-concentrating hormone type 1 receptor (MCH1R) KO mice obtained from Merck (1) were used for cocaine induced conditioned place preference (CPP) experiments as well as cocaine sensitization experiments. MCH1R KO mice were backcrossed to a C57BL/6 backgrounds at least for 9 generations. Male C57BL/6 mice (National Cancer Institute), age 9–11 weeks were used for the locomotion study to measure the effect of MCH on cocaine. Male Sprague–Dawley (Charles River) rats (350–400 g) were used for cocaine self-administration and in situ hybridization study. Male postnatal day 23–26 Sprague–Dawley (Charles River) rats were used for slice preparation for immunoblotting. Rats used for cocaine self administration were single housed. All of the other animals were group-housed and maintained on a 12-h light/dark cycle (lights on at 07:00) with food and water available ad libitum. All experimental procedures were performed in compliance with National Institutes of Health Guide for Care and Use of Laboratory Animals, and approved by the local Institutional Animal Care and Use Committee.

Drugs. Cocaine HCl (Sigma-Aldrich) and Yohimbine HCl (Sigma-Aldrich) was dissolved in sterile saline solution (NaCl, 0.9%). MCH (Bachem) and TPI 1361-17 (2) was dissolved in saline solution. SKF 81297 (Tocris) and Quinpirole (Sigmal-Aldrich) was dissolved in DMSO as stock solution, and diluted in artificial cerebro-spinal fluid (aCSF) before experiments.

Double in Situ Hybridization. Rat MCH-1 receptor cDNA was subcloned into pBluescript SK (Stratagene). The probe was digested with either EcoRI or XhoI, and then antisense and sense [^{35}S]-UTP-labeled riboprobes were synthesized by T3 and T7 RNA polymerases, respectively (Amersham). The Dopamine 1 receptor probe was digested with either HindIII or *EcoR1*, and then antisense and sense digoxigenin (DIG) UTP-labeled riboprobes were synthesized by T7 and SP6 RNA polymerases, respectively, by using a DIG RNA labeling kit (Roche Applied Science). The Dopamine 2 receptor probe was digested with either *EcoR1* or *XbaI1*, and then antisense and sense DIG UTP-labeled riboprobes were synthesized by T7 and SP6 RNA polymerases, respectively, by using a DIG RNA labeling kit. Vectors containing Dopamine D1 and D2 receptor were provided by Dr. Watson (University of Michigan). All probes were separated from unincorporated nucleotides on Sephadex G-50 columns (Roche Applied Science).

Tissue sections were processed for in situ hybridization as previously described (3) with slight modifications. Briefly, sections were pretreated with proteinase K (0.1 μg/mL), acetylated, dehydrated through ethanol (50, 70, 95, and 100%), and air dried. Pretreated sections were then incubated for 20 h at 60 °C, with hybridization buffer (50% formamide/10% dextran sulfate/0.02% Ficoll/0.02% polyvinylpyrolidone/0.02% BSA/500 μg/mL tRNA/10 mM DTT/0.3 M NaCl/10 mM Tris, pH 8.0/1 mM EDTA, pH 8.0) containing [^{35}S]-UTP labeled sense or antisense riboprobes (10^7 cpm/mL). After the sections were hybridized, they were treated with RNase A (20 μg/mL) for 30 min at 37 °C, and then washed 4 times in decreasing salinity (from 2 to 0.1× SSC buffer) and a 30-min wash at 68 °C. Next, sections were dehydrated through ethanol (50, 70, 95, and 100%), air dried, and exposed to MR-1 Kodak film for 3–6 days. After the film was developed, sections were dipped in photographic NTB-2 emulsion (Kodak). After 3–6 weeks, slides were developed in 1:1 diluted Kodak D-19, fixed, coverslipped, and analyzed under a microscopy (BX50, Olympus).

Double in situ hybridization was carried out as described previously (4) with modification. Briefly, stock concentrations of DIG-labeled D1, or D2 receptor riboprobes were determined by using dot blot hybridization, followed by optical density measurements. To determine the optimal working concentrations for the various riboprobes, each riboprobe was diluted serially with hybridization buffer and hybridized to test sections first. For each probe, 1 concentration giving the strongest signal at minimal background was chosen for the experimental sections. For experimental sections, a combination of isotopic and colorimetric in situ hybridization was used. First, sections were prehybridized as described above. Then, DIG-labeled riboprobes of D1 receptor (0.5 mg/mL) or D2 receptor (0.5 mg/mL) mixed with ^{35}S-labeled MCH1 receptor riboprobe (2×10^7 cpm/mL) in hybridization buffer and incubated with sections at 60 °C overnight. After hybridization, sections underwent the same posthybridization process as described above. Final stringent washes were for 30 min in 0.1 × SSC at 68 °C. After incubation with 0.1× SSC, sections were rinsed in Genius Buffer 1 (GB1; 100 mM Tris-HCl/150 mM NaCl, pH 7.5) for 1 min, and then incubated with blocking buffer containing GB1, 0.25% Triton X-100, 10% horse serum for 1 h at room temperature. Alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche Diagnostics) was diluted 1:2,500 in blocking solution containing 3% horse serum, and 500 μL was applied to each slide, followed by incubation at 4 °C for overnight. Tissues were then washed and incubated with color substrate nitroblue tetrazolium salt (0.5 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.187 mg/mL) in GB3 (100 mM Tris·HCl/100 mM NaCl/50 mM $MgCl_2$, pH 9.5) in the dark for 12–16 h at room temperature. Sections were washed again and air dried overnight before being exposed to film as described above. After the films were developed, slides were dipped in 0.3% parlodin dissolved in isoamylacetate for 5 s, and dried overnight. Last, slides were dipped in photoemulsion and processed as described above.

Slice Preparation and Electrophysiology. Adult (days 55–90), male, Sprague–Dawley rats were deeply anesthetized with 40 mg/kg pentobarbital (i.p.) and perfused transcardially with ≈30 mL of chilled modified aCSF at a rate of ≈10 mL/min. The modified aCSF for perfusion contained (in mM): 225 sucrose; 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 4.9 $MgCl_2$, 0.1 $CaCl_2$, 26.2 $NaHCO_3$, 1.25 glucose; 1 ascorbic acid and 3 kynurenic acid. The brain was removed rapidly, and coronal slices (300 μm) containing the NAcb were cut in this same modified aCSF. Slices recovered at 32 °C in carbogen-bubbled aCSF (126 mM NaCl/2.5 mM KCl/1.2 mM NaH_2PO_4/1.2 mM $MgCl_2$/2.4 mM $CaCl_2$/18 mM $NaHCO_3$/11 mM glucose, pH 7.2–7.4/mOsm 301–305) for 30 min to 5 h, with 1 mM ascorbic acid added just before placing slices in the recovery chamber. During experiments, slices were submerged and continuously perfused (≈2 mL/min) with carbogen-bubbled aCSF warmed to 31–32 °C, and supplemented with CNQX (10 μM, to block AMPA-type glutamate receptors) and picrotoxin (50 μM, to block GABA-A receptors).

Electrophysiology experiments were performed as described (5) using whole-cell recording and visualized infrared-DIC with a potassium methanesulfonate-based internal solution containing (in mM): 130 KOH, 105 methanesulfonic acid, 17 hydrochloric acid, 20 Hepes, 0.2 EGTA, 2.8 NaCl, 2.5 mg/mL MgATP, and 0.25 mg/mL GTP, pH 7.2–7.4, 280–290 mOsm. Electrical

signals were be recorded by using Clampex 9.2 and an Axon 700A patch amplifier (Axon Instruments). After breaking into a neuron, the resting membrane potential was set to ≈−80 mV by injecting DC current through the patch amplifier. To measure firing, current pulses were applied using a patch amplifier in current-clamp mode, with a series of 7–8 current pulses (300-ms duration) applied every 30 s, so that the minimum current amplitude for each cell was just subthreshold for spike firing. Depolarizing pulses were alternated with a 33.3 pA hyperpolarizing pulse to examine input resistance. Agonists were bath applied for 8 min, and the change in firing determined for the current step that gave 4 or 5 action potentials before addition of the receptor agonist(s). TPI 1361-17 was applied 5 min before MCH and dopamine receptor agonists.

Slice Preparation for Immunoblotting. Slices were prepared from male postnatal day 23–26 Sprague–Dawley rats. After decapitation, brains were rapidly fixed to the cutting surface of a Vibratome (Electron Microscopy Sciences), and coronal slices (350 μm) were cut. NAcSh slices were cut from the coronal sections, and slices were recovered at 30 °C in oxygenated (95% O_2 and 5% CO_2) artificial CSF (in mM/126 NaCl/1.6 KCl/1.2 NaH_2PO_4/1.2 $MgCl_2$/2.4 $CaCl_2$/18 $NaHCO_3$/11 glucose, pH 7.2–7.4) for 1 h. At the end of the incubation period, slices were incubated with agonists for 5 min. After treatment, the slices were immediately frozen on dry ice.

PhosphoThr-34 DARPP-32 was assayed by Western blotting as described previously (6) by using anti-phospho-Thr 34 DARPP-32 antibody (dilution 1:1,000; gift from Dr. Paul Greengard) (7, 8). A monoclonal antibody generated against DARPP-32, which is not phosphorylation state specific, was used to estimate the total amount of DARPP-32.

Locomotor Sensitization. In the locomotor sensitization assay, locomotor activity was monitored for 15 min after an i.p. saline injection (days 1–2), then after daily cocaine injections (12 mg/kg; days 3–7).

Determination of Phospho-DARPP-32 After Chronic Cocaine Injections. Experiments were performed as described in locomotor sensitization assay. On day 7 of treatment, mice were killed rapidly by decapitation 30 min after injection of either vehicle or cocaine. The brains were rapidly removed and the NAcSh were rapidly dissected out on an ice cold surface. Immunoblotting was performed as described above using anti-phospho-Thr 75 DARPP-32 antibody (dilution 1:5,000; gift from Dr. Paul Greengard) (6, 7)

Cocaine Induced CPP. A 2-chambered Plexiglas box (15 × 7 × 12 inch per chamber) was used. One compartment consisted of black and white striped walls with lemon scent applied and white paper bedding. The other compartment had polka dots, banana scent, and woodchip bedding. A sliding door separated the 2 boxes closed with a black cover. On day 1, preconditioning was conducted. Mice were given access to both chambers for 20 min. Their activity was measured, and the time spent in each chamber was recorded as the preconditioning chamber preference. Mice that spent ≈25% more time in any chamber compared with the other were eliminated from the study. On days 2 to 7, drug conditioning was performed. On alternate days, mice were injected either with saline or cocaine (6, 12, and 18 mg/kg, i.p.), and confined for 15 min in each compartment and access to the other chamber was blocked. On day 8, place preference was determined by giving mice access to both chambers for 20 min, and the activity was monitored. No injections were given before this test. Place preference score was determined by subtracting the amount of time spent on the saline side from the time spent on the cocaine side during post conditioning test. Activity was measured by using a VERSAMEX system.

Surgery. Catheter construction has been described previously (9). Animals were anesthetized with ketamine and xylazine (100 and 10 mg/kg each, i.p.), and a chronic catheter was surgically implanted into the external jugular vein. The catheter was passed from the animal's back to the jugular vein, where it was implanted. The catheters were flushed daily with 0.2 mL sterile heparinized saline solution (20 units heparin/mL of saline) to maintain catheter patency. On test days, heparinized saline was injected before and after the self-administration session.

Intracerebroventricular cannulation was done as described before (10). Animals were placed in a stereotaxic instrument (Kopf Instruments). By using aseptic procedures, a midline incision was made, the skin overlying the skull was retracted, and 3 small holes were drilled in the skull. Stainless steel anchor screws were screwed into the adjacent holes, and a guide cannula (Plastics One) was lowered through the center hole to a predesignated spot in the lateral ventricle according to the following coordinates from the atlas of Paxinos and Watson (11): anteroposterior (AP), −0.92 mm; mediolateral (ML), +1.4 mm; and dorsoventral (DV), −3.4 mm. Dental cement was applied such that the exposed skull was completely covered, and the cannula was securely fastened to the screws. After the cement dried (3–5 min), the wound was closed with wound clips, an antiseptic ointment was applied, and a dummy cannula was placed in the cannula to prevent clogging.

NAcSh and NAcCo cannulation was done the same as for the i.c.v. cannulation, except that different coordinates were used for NAcSh and NAcCo. Coordinates for placement of the guide cannula 1.0 mm above the injection point in the NAcSh were as the followings: AP, +1.7 mm; ML, −0.75 mm; and DV, −5.4 mm. The coordinates for NAcCo were: AP, +1.7 mm; ML, +2.5 mm; and DV, −5.9 mm.

Cocaine Self-Administration. Cocaine self-administration experiments were conducted in ventilated, sound attenuating operant chambers (Med Associates). Animals were placed in the chamber (28 × 25 × 30 cm) with 2 nose poke holes installed on the wall. A 10-mL glass syringes was mounted in an infusion pump outside of the chamber, and connected through polyethylene tubing to the cannula assembly on the animal's back. The syringe was filled with cocaine solution to deliver a fixed i.v. dose of cocaine (500 μg/kg per injection).

Self-administration under a fixed ratio (FR)1 schedule. After 4 daily sessions of cocaine self-administration, the effect of TPI 1361-17 was tested. On the test day, the conditions were identical, except that rats were injected either with vehicle or with various concentrations of TPI 1361-17 (5, 10, or 20 nmol i.c.v.) 5 min before the session. After the final daily test session, propofol (0.6 mL/kg) was injected through the catheter to test the patency of the i.v. catheter, as indicated by rapid (5–10 s) anesthesia. Data from all animals not demonstrating rapid anesthesia were discarded.

Self administration under a progressive ratio (PR) schedule. Initial cocaine self-administration under FR1 schedule (500 μg/kg per injection) was the same as described above. After stable cocaine self-administration was established, the rats were switched to cocaine self-administration under a PR schedule. During the PR schedule, the number of nose pokes required to get an i.v. cocaine infusion was progressively increased within each test session (12), according to the following PR series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, and 603, according to Response ratio = $[5e^{(\text{injection no.}\times 0.2)}]-5$. The break point was defined as the number of completed nose poking completed for the last cocaine infusion before 30 min, during which no infusions were

obtained by the rat. Break point (percentage baseline) was calculated as percentage changes of breakpoint after vehicle or TPI 1361-17 injection compared with 2 previous sessions, and finally normalized to vehicle group. Sessions lasted until the rat failed to get an infusion for 30 min or for a maximum of 4 h. Rats were allowed to continue cocaine self-administration under the PR session until day-to-day variability in break points did not vary by >2 ratios for 3 consecutive days. Once a stable base line was established, rats received either vehicle or TPI 1361-17 before the session in a random order. Each rat received all treatments, and the order of administration of drug or vehicle for any single rat was determined randomly with approximately half receiving drug first. At least 2 days of baseline cocaine self-administration separated each test. Data from animals not maintaining i.v. catheter were discarded.

Microinjection Procedure. The rats were gently hand held while the injectors were inserted. The injectors for i.c.v. injection were 0.2 mm longer than the guide cannulas, and the injectors for NAcSh and NAcCo were 1 mm longer than guide cannulas. Once the injectors were inserted, the rats were placed into an open cage and allowed to move freely during the infusion. The infusion pumps (CMA, MA) delivered 5 μL/4 min of drug or vehicle for i.c.v. injection or 0.5 μL/min of drug or vehicle/side for NAcSh and NAcCo injections. The injectors were left in the brain for 1 additional minute after the end of injection before being removed.

Cue-, Cocaine-, and Stress-Induced Reinstatement. In reinstatement experiments, rats were trained to self-administer cocaine under an FR-1 schedule of reinforcement for 2-h daily sessions for 10–12 days as described above, after which the extinction period followed. During the extinction phase, cocaine was not available, and the cues previously associated with cocaine availability (the house light and cue lights) were not presented. Response in each nose poke had no consequences. Extinction sessions continued until response reached an extinction criterion for 3 consecutive days of <25% of their baseline responses during cocaine self-administration at the previously active hole. Reinstatement was induced by exposing animals to cue, cocaine injection (10 mg/kg i.p.), or yohimbine injection (2.5 mg/kg i.p.)

Cue Induced Reinstatement. On the test days, responding in an active hole resulted in the illumination of cue light for 5.6 s without delivering cocaine solution. A house light was on during the reinstatement session. Rats were given either with vehicle or TPI 1361-17 10 nmol (i.c.v.) 5 min before the session and responding in an active hole as well as inactive hole was counted for 2 h.

Cocaine Induced Reinstatement. On the test days, rats were given 10 mg/kg of cocaine (i.p.) just before they were placed in the test chamber. During the cocaine-induced reinstatement session, cocaine was not available, and the cues were not presented. Nose poking in the absence of cues and cocaine infusions was measured as the relapse behavior. Vehicle or TPI 10 nmol was given 5 min before the cocaine injection.

Stress-Induced Reinstatement. On the test days, rats were given yohimbine (2.5 mg/kg, i.p.) just before they were placed on the test chamber. During the stress-induced reinstatement session, cocaine was not available, and the cues were not presented. Nose poking in the absence of cues and cocaine infusions was measured as the relapse behavior. Vehicle or TPI 10 nmol was given 5 min before the yohimbine injection.

Data Analysis. Prism software (GraphPad) was used for statistical analysis. Data expressed as mean ± SEM. Results were analyzed by *t* test or ANOVA followed by the appropriate post hoc comparisons, and $P < 0.05$ was considered statistically significant.

1. Marsh DJ, et al. (2002) Melanin-concentrating hormone 1 receptor-deficient mice are lean, hyperactive, and hyperphagic and have altered metabolism. *Proc Natl Acad Sci USA* 99:3240–3245.
2. Nagasaki H, et al. (2008) The pharmacological properties of a novel MCH(1) receptor antagonist isolated from combinatorial libraries. *Eur J Pharmacol* 602:194–202.
3. Winzer-Serhan UH, Leslie FM (1997) Codistribution of nicotinic acetylcholine receptor subunit alpha3 and beta4 mRNAs during rat brain development. *J Comp Neurol* 386:540–554.
4. Xu YL, et al. (2004) Neuropeptide S: A neuropeptide promoting arousal and anxiolytic-like effects. *Neuron* 43:487–497.
5. Hopf FW, et al. (2003) Cooperative activation of dopamine D1 and D2 receptors increases spike firing of nucleus accumbens neurons via G-protein betagamma subunits. *J Neurosci* 23:5079–5087.
6. Reinscheid RK, et al. (2005) Pharmacological characterization of human and murine neuropeptide s receptor variants. *J Pharmacol Exp Ther* 315:1338–1345.
7. Snyder GL, et al. (1992) Phosphorylation of DARPP-32 and protein phosphatase inhibitor-1 in rat choroid plexus: Regulation by factors other than dopamine. *J Neurosci* 12:3071–3083.
8. Bibb JA, et al. (1999) Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature* 402:669–671.
9. Manzardo AM, Stein L, Belluzzi JD (2002) Rats prefer cocaine over nicotine in a two-lever self-administration choice test. *Brain Res* 924:10–19.
10. Jenck F, et al. (1997) Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress. *PNAS* 94:14854–14858.
11. Paxinos G, Watson C (1998) *The Rat Brain in Stereotaxic Coordinates* (Academic, San Diego, CA), 4th Ed.
12. Richardson NR, Roberts DC (1996) Progressive ratio schedules in drug self-administration studies in rats: A method to evaluate reinforcing efficacy. *J Neurosci Methods* 66:1–11.

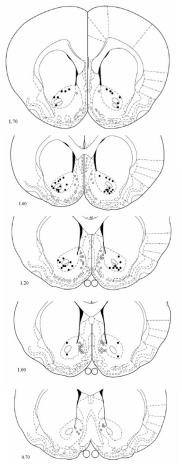

Fig. S1. Injection placements for NAcSh (○) and NAcCo (●) for Fig. 5C.