Supporting Information

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SI Materials and Methods

Drug Injections and Histological Analysis. Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD) was dissolved in saline (0.25 mg/0.1 mL) and given i.v. SB-334867 (Tocris) was dissolved in 10% (vol/vol) 2-hydroxypropyl-β-cyclodextrin, 1% (vol/vol) DMSO, diluted with sterile water and given i.p. 30 min before test. The NPS antagonist SHA 68 (3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide) was synthesized at National Institutes of Health. It was dissolved in 10% cremophor EL, 4% DMSO, and 86% of distilled water and given i.p. 30 min before test. NPS and [D-Cys (tBu) (5)]NPS, kindly provided by Dr. R.Guerrini (University of Ferrara, Italy), were dissolved in saline and given ICV (1 μL/rat) or into brain areas (0.3 μL/site). Peptides were injected immediately before placing the animals into the self-administration chambers. At completion of experiments, after staining with cresyl violet, sections were examined under a microscope for the location of injector tip placement. An expert observer, blind to treatment conditions and behavioral data, did the histological verification. The analysis revealed that three animals (two of the LH and one of the PeF group) had one of the two cannulae placed between the LH and the PeF. Another rat of the LH group had one cannula located too anterior (approximately AP 1.88). These animals responded to NPS injection. Four rats belonging to the DMH group had the cannulae bilaterally placed too high with respect to the target area. These animals did not respond to NPS injection. Data obtained in all these rats were excluded from statistical analysis. ICV and CeA cannulae were in the correct position in all of the animals.

Histochemistry. ***Tissue preparation.*** Rats were deeply anesthetized by CO_2 inhalation and transcardially perfused with 4% paraformaldehyde in PBS.

Brains were postfixed for 1 h and cryoprotected in 30% sucrose solution in PBS at 4 °C until sectioning (30-μm thick for single and double immunohistochemistry, 40-μm thick for in situ hybridization combined with immunohistochemistry). All reactions were carried out on free-floating sections as previously described (1).

c-Fos immunostaining. Sections were rinsed in PBS, treated 30 min in 1% H_2O_2 in PBS to reduce background and incubated in blocking solution [3% normal sheep serum (NSS) in PBS] for 2 h. Sections were then placed in a solution (3% NSS in PBS) containing an antibody directed against c-Fos (1:10,000; Ab-5; Calbiochem) overnight at room temperature (RT). Following several washings in PBS, sections were incubated in blocking solution (3% NSS in PBS) containing biotinylated goat anti-rabbit antibody (1:500; Vector Laboratories) for 90 min followed by 1 h incubation in an avidin-biotin peroxidase complex (ABC) solution (Vector Laboratories). Immunoreactivity was visualized as a black reaction product after 7 min in a 0.04% 3,3′ diaminobenzidine tetrahydrochloride (DAB) solution containing 0.01% H_2O_2 (Sigma) and 0.1% nickel ammonium sulfate. Sections were mounted on Superfrost1 slides (Fisher Scientific), air dried, dehydrated by serial alcohol rinsing, cleared in xylene, and coverslipped.

Double immunostaining for c-Fos and Hcrt-1/Ox-A. Sections were rinsed in PBS, treated 30 min in 1% H_2O_2 in PBS, and incubated in blocking solution (3% donkey serum in PBS) for 2 h. Sections were placed in blocking solution (3% donkey serum in PBS) containing antibodies directed against c-Fos (1:10,000; Ab-5; Calbiochem) and Orexin-A (1:1,500; Santa Cruz Biotechnology) overnight at RT. After several washings in PBS, sections were incubated in blocking solution (3% donkey serum in PBS) containing biotinylated donkey anti-rabbit antibody (1:500; Jackson Immunoresearch Laboratories) for 90 min. Sections were then processed in ABC solution (Vector Laboratories) for 1 h, and immunoreactivity could be visualized as a black reaction product after 7 min in a 0.04% 3,3′ diaminobenzidine tetrahydrochloride (DAB) solution containing 0.01% H_2O_2 and 0.1% nickel ammonium sulfate. Sections were then incubated in blocking solution (3% donkey serum in PBS) containing biotinylated donkey anti-goat antibody (1:500; Jackson Immunoresearch Laboratories) for 90 min and in ABC solution (Vector Laboratories) for 1 h. The immunoreactivity could be visualized as a brown reaction product after 2 min detection in a 0.04% 3,3′ diaminobenzidine tetrahydrochloride (DAB) solution containing 0.01% H_2O_2.

Cell counting. c-Fos–like immunoreactivity was assessed by light microscopy (Leica DMR light microscope) at a magnification of ×50. The number of c-Fos–positive nuclei was counted bilaterally with the use of the ImageJ analysis software system (2) marking the cells with the pointer tool to avoid miscounting. Two sections for each rat were counted bilaterally in the following subregions (from −1.8 mm to −3.6 mm from Bregma): medial amygdala (MeA), central amygdala (CeA), lateral hypothalamus (LH), perifornical nucleus (PeF), dorsomedial (DM), ventromedial (VMH), and arcuate nuclei of hypothalamus (Arc), caudate putamen (Cpu), and ventromedial thalamic nucleus (VM). Regions were selected by the free-hand selection tool in ImageJ according to anatomical landmarks as in ref. 2.

c-Fos and Hcrt-1/Ox-A immunoreactivity was assessed by light microscopy. Double labeled cells were easy to identify as Hcrt-1/Ox-A cell bodies were stained brown and c-Fos positive nuclei were stained black. The number of c-Fos positive nuclei in the Hcrt-1/Ox-A neurons was counted bilaterally with the use of the ImageJ analysis software system marking the cells with the pointer tool to avoid miscounting. Two sections were counted for each rat bilaterally in the following subregions (from −1.8 mm to −3.6 mm from Bregma): LH, PeF, and DMH. Regions were selected by the free-hand selection tool in ImageJ according to anatomical landmarks as in ref. 2. Data are expressed as the percentage of Fos-positive neurons that also stained for Hcrt-1/Ox-A and percentage of Hcrt-1/Ox-A stained cells that are also Fos positive.

Colocalization of Hcrt-1/Ox-A immunoreactivity and NPSR transcript. The NPSR cRNA probe was generated with the following forward and reverse primers: 5′-AAGTCTCACATTTATTTTCA-3′ and 5′-TCTACTGCATCTTCAGCAGC-3′, respectively. The region bounded by these primers was 508 bases long and was complementary to nucleotides 1076–1583 of the mouse NPSR mRNA (accession no. NM_175678). PCR products were subcloned into pBluescript vector using standard techniques. The identities and directionalities of the inserts were confirmed by DNA sequencing. To synthesize digoxigenin (DIG)-labeled antisense cRNAs, the plasmids were linearized by restriction and then subjected to in vitro transcription with T3 RNA polymerase and DIG-labeled UTP (Roche Diagnostics) according to the manufacturer's protocol (Ambion).

Free-floating sections were incubated in PBS supplemented with 0.2% Triton X-100 for 10 min, deproteinized with 0.2 N HCl for 10 min and acetylated with acetic anhydride (0.25% in 0.1 M triethanolamine hydrochloride, pH 8) at RT for 10 min. After postfixation for 10 min in 4% paraformaldehyde, sections were prehybridized in a humidified chamber at 56 °C for 2 h in a solution containing: 4× piperazine-N,N′-bis[2-ethanesulfonic acid] (Pipes), 10% (wt/vol) dextran sulfate, 50% deionized formamide, 5×

Denhardt's, 50 mM DTT (DTT), 2% SDS (SDS), 250 μg/mL denatured herring sperm DNA, and 250 μg/mL yeast tRNA. Antisense DIG-labeled cRNA probe (20–80 pg/μL) was added to the sections for an overnight incubation at 56 °C. The sections were then transferred into new vials and washed with 2× SSC supplemented with 10 mM β-mercaptoethanol (β-ME) for 30 min at RT, digested with 4 μg/mL RNase A in a solution containing 50 mM Tris-HCl (pH 8), 5 mM EDTA and 0.5 M NaCl at 37 °C for 1 h, rinsed in 1× SSC, 50% deionized formamide, and 5 mM β-ME at 56 °C for 2 h, and washed in 0.2× SSC, 1 mM β-ME, and 0.5% Sarkosyl (sodium lauryl sarcosinate, Sigma) at 65 °C for 1 h, followed by 3× 5 min Tris-buffered saline (TBS; pH 7.5) washes at RT. After 1 h incubation at RT in a solution containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 4% BSA, sections were incubated with a sheep anti-DIG antibody conjugated to alkaline phosphatase (1:1,000, Roche Diagnostics) overnight at 4 °C. Sections were then washed with TBS (5× 5 min) and immersed 2× 10 min in a solution of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$ (NTM) supplemented with 0.5 mg/mL Levamisol (Vector Laboratories) to inactivate endogenous alkaline phosphatase activity. Colorimetric revelations were made with nitroblue tetrazolium (NBT) plus 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP, Roche Diagnostics) to produce a dark purple coloration in NPSR expressing neurons. Intermittent microscopic observations allowed determination of time conditions yielding optimal signal to noise ratio (4 h to overnight incubations). The reaction was stopped with 5× 10-min rinses in TBS.

Finally, to detect hypocretin cells, we combined an immunohistochemistry using anti-Hcrt1/Orex-A antibodies (1/750; C-19 no. sc-8070, Santa Cruz Biotechnology) following the protocol described below. Sections were incubated overnight at 4 °C with the anti-Hcrt1/Orex-A antibody in a blocking buffer containing PBS, 4% BSA, and 0.1% Triton X-100. After several rinses in PBS supplemented with 0.1% Triton X-100, sections were incubated for 2 h with biotinylated anti-goat IgG secondary antibodies (1:400 dilution with ABC Vectastain Elite kit, Vector Labs). Then, further rinses were followed by incubation in avidin-biotin-horseradish-peroxidase solution (ABC Vectastain Elite kit, Vector Labs) for 1 h. Hcrt-1/Orex-A neurons were dyed brown after incubation in a Tris-HCl solution (0.125 M, pH 7.5) containing 0.04% of diaminobenzidine (DAB, Sigma) and supplemented with increasing concentrations of H_2O_2 (from 0.00015 to 0.0048%; Sigma). Afterward, the reaction was stopped by three 10-min rinses in Tris-HCl (0.125 M, pH 7.5). Tissue sections were mounted onto SuperFrost Plus slides (Fisher Scientific), and dehydrated in ethanol, cleared in Clear-Rite 3, and coverslipped with Vectamount mounting medium (Vector Labs). Sections were analyzed using standard light microscopy with an Axioplan microscope (Zeiss).

1. Ciccocioppo R, Sanna PP, Weiss F (2001) Cocaine-predictive stimulus induces drug-seeking behavior and neural activation in limbic brain regions after multiple months of abstinence: Reversal by D(1) antagonists. *Proc Natl Acad Sci USA* 98:1976—1981.

2. Paxinos G, Watson C (1998) *The Rat Brain in Stereotaxic Coordinates* (Academic Press, San Diego), 4th Ed.

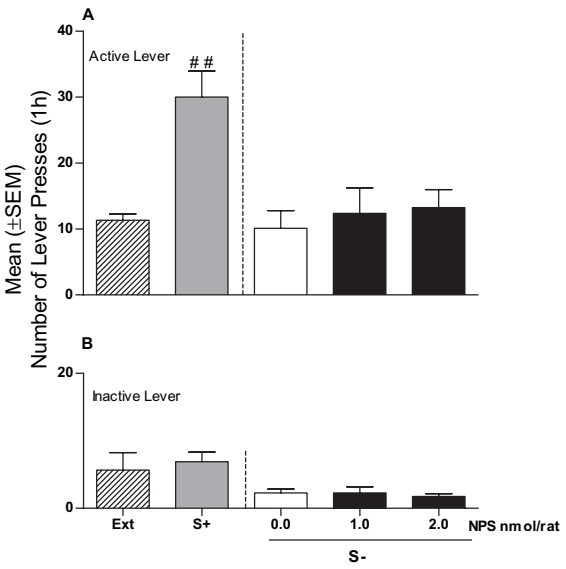

Fig. S1. Effects of ICV injection of NPS (0.0, 1.0, and 2.0 nmol/μL/rat) on cue-induced reinstatement of responding for saline-paired cues (S^-). Reinstatement responses in rats ($n = 8$) exposed to S^+ and S^- condition (in the absence of reward delivery) compared with the mean number of lever presses of the last 3 d of extinction (EXT). (*A*) Mean (±SEM) number of responses at the previously active lever; (*B*) mean (±SEM) number of responses at the inactive lever. Different from extinction, $^{\#\#}P < 0.01$.

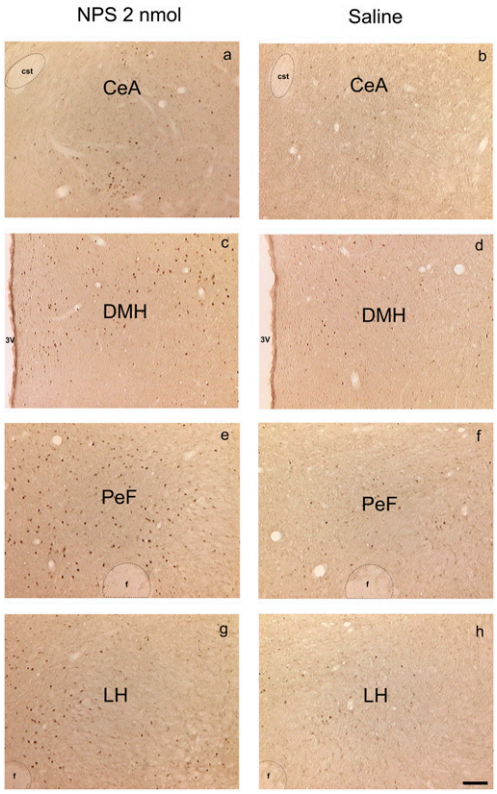

Fig. S2. Photomicrographs showing c-Fos–ir cells in the (*A*) central amygdala (CeA), (*C*) dorsomedial hypothalamic nucleus (DMH), (*E*) perifornical nucleus (PeF), and (*G*) lateral hyphothalamus (LH) of rats ($n = 4$) after icv NPS (2 nmol) injection. Photomicrographs showing c-Fos–ir cells in the (*B*) CeA, (*D*) DMH, (*F*) PeF, and (*H*) LH after icv saline injection. Sections were taken between −1.8 mm and −3.6 mm from Bregma according to ref. 2. (Scale bar, 100 μm.) cst, commissural stria terminalis; f, fornix; 3V third ventricle.

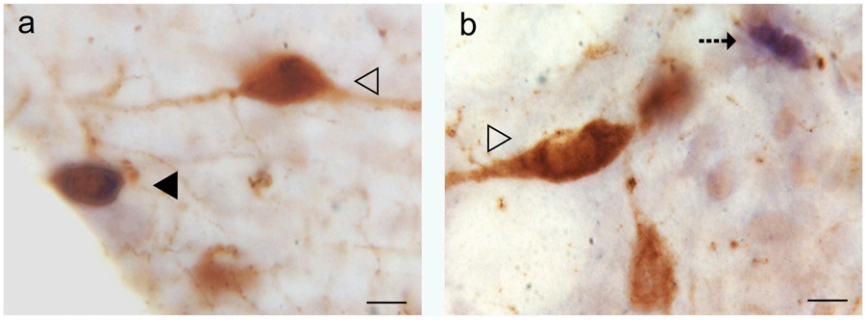

Fig. S3. Photomicrograph of dual labeling for NPSR mRNAs (dark purple) and Hcrt-1/Ox-A immunoreactivity (brown). The black arrow indicates a hypocretinergic cell expressing NPSR mRNA (*B*), the white arrows indicate and a Hcrt single-labeled neuron (*A* and *B*) and the broken arrow indicates a NPSR single-labeled neuron adjacent to a hypocretinergic neuron (*B*). (Scale bar, 10 μm.)

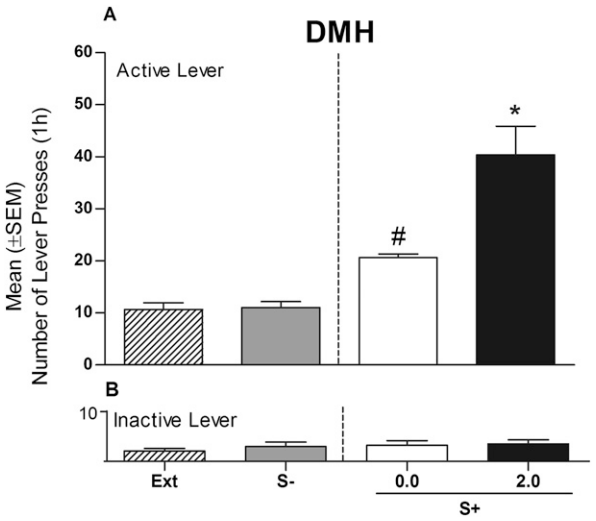

Fig. S4. Effect of intracranial bilateral intra-DMH injection of a high dose of NPS (2.0 nmol) or its vehicle (0.0) on cue-induced reinstatement of cocaine seeking. Mean (± SEM) number of responses at the: (*A*) cocaine active and (*B*) inactive levers. Different from extinction, $^{\#}P < 0.05$; different from vehicle, $^{*}P < 0.01$.

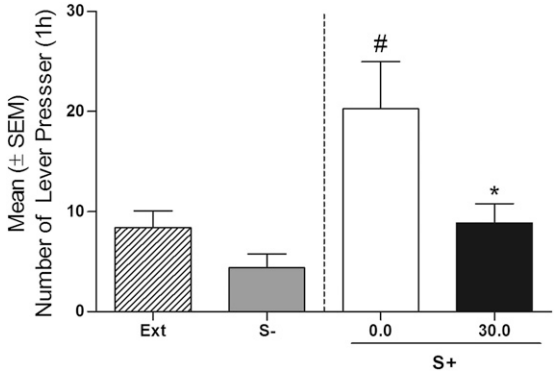

Fig. S5. Effects of LH injection of [D-Cys(tBu) ⟨5)]NPS (0.0 and 30 nmol/μL/rat) on cue-induced reinstatement of responding for cocaine paired cues (S^{+}). Reinstatement responses in rats ($n = 8$) exposed to S^{+} and S^{-} condition (in the absence of reward delivery) compared with the mean number of lever presses of the last 3 d of extinction (EXT). (*A*) Mean (±SEM) number of responses at the previously active lever; (*B*) mean (±SEM) number of responses at the inactive lever. Different from extinction, $^{\#}P < 0.05$. Different from vehicle (0.0), $^{*}P < 0.05$.

Table S1. Number of c-Fos–positive cells following ICV injection of NPS or vehicle

Brain region	2 nM NPS	Vehicle
CeA	96.0 ± 2.7**	41.0 ± 0.6
MeA	113.0 ± 3.7**	44.0 ± 5.6
LH	92.0 ± 4.0**	38.0 ± 1.5
PeF	134.0 ± 2.0**	35.0 ± 0.6
VMH	136.0 ± 5.8**	58.0 ± 4.2
DMH	273.0 ± 13.1**	64.0 ± 3.0
Arc	271.0 ± 6.3**	95.0 ± 1.6
CPu	26.0 ± 1.5	24.0 ± 2.6
VM	36.0 ± 2.5	44.0 ± 3.8

Values are mean ± SEM of c-Fos–positive cells/mm^2. Sections were taken between −1.8 mm and −3.6 mm from Bregma according to ref. 2. The following subregions were analyzed: central amygdala (CeA), medial amygdala (MeA), lateral hyphothalamus (LH), perifornical nucleus (PeF), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), arcuate hypothalamic nucleus (Arc), caudate putamen (Cpu), and ventromedial thalamic nucleus (VM). $^{**}P < 0.01$ compared with vehicle group.

Table S2. Percentage of c-Fos–positive Hcrt-1/Ox-A cells after ICV NPS or saline

Area	2 nM NPS $n = 4$	Saline $n = 4$
LH	42.3 ± 0.7	10.5 ± 0.5
PeF	43.8 ± 0.6	10.3 ± 0.6
DMH	22.5 ± 2.7	15.3 ± 0.6

Values are mean ± SEM of the percentage of Fos-positive Hcrt-1/Ox-A cells. Sections were taken between −1.8 mm and −3.6 mm from Bregma according to ref. 2. The following subregions were analyzed: lateral hypothalamus (LH), perifornical nucleus (PeF), and dorsomedial hypothalamic nucleus (DMH).