Supplemental materials

Supplemental methods

Subjects. Original C57/BL6 breeding pairs were purchased from Jackson Laboratories (Bar Harbor, Maine). All animals used in the study were bred at New York University's animal facility, and all procedures relating to the use of animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of New York University (A3317-01). All animals were kept on a 12/12 light–dark cycle, with lights going out at 7 pm. Water was always available *ad libitum*. Food was available *ad libitum*, except when specified.

Stereotaxic surgery for viral injection. At postnatal day (P) 26 ($\pm$ 3), mice were bilaterally injected with viruses. For bidirectional control of mPFC pyramidal cell activity, the mPFC of 20 female mice were injected with 100nL per hemisphere of a 50/50 mixture of pAAV8-CaMKIIα-hM3D(Gq)-mCherry (gift from Bryan Roth; Addgene viral prep # 50476-AAV8; RRID: Addgene_50476; viral titer of 3×10^{12} vg/mL) and AAV8-DJ-CaMKIIα-HA-KORD-IRES-mCitrine (Stanford University Gene Vector and Virus Core, Stanford, CA; viral titer of 2.1×10^{12} vg/mL). For bidirectional control of the mPFC-to-DS pathway, mPFC of 9 female mice were injected with 100nL of a 50/50 mixture of AAV1-hSYN1-dF-HA-KORD.IRES.mCitrine (gift from Dr. Yavin Shaham of NIDA, produced by the Genetic Engineering and Viral Vector Core of NIDA; viral titer of $2x10^{12}$ vg/mL) and AAV8-hSYN-DIO-hM3D(Gq)-mCherry (gift from Bryan Roth; Addgene viral prep # 44361-AAV8; RRID: Addgene_44361; viral titer of 4×10^{12} vg/mL). DS of the same animals

was injected with 400-500nL of AAV-rg-pmSyn1-EBFP-Cre (gift from Hongkui Zeng; Addgene viral prep # 51507-AAVrg; RRID: Addgene_51507; viral titer of 6×10^{12} vg/mL).

As described under 'Experimental design', three types of control subjects were used. GFP-CON mice (N=5) received 100nL of the control virus pAAV-CaMKIIα-eGFP (gift from Bryan Roth; Addgene viral prep #50469-AAV8; RRID: Addgene_50469, 1×10^{13} vg/mL), to express GFP in CaMKIIα+ pyramidal cells without co-expression of DREADD virus. No-cre-CON mice (N=6) received 100nL of the 50/50 mixture of both cre-dependent DREADD viruses into the mPFC, but no retrograde cre virus was delivered. No-Drug-Cre-CON mice (N=5) received 200nL of cre virus AAV5.CMV.H1.eGFP-Cre.PRES.SV40 (gift from James M. Wilson; Addgene viral prep #105545, RRID:Addgene_105545, viral titer of 7×10^{12}) in a control region, the dorsal hippocampus.

Surgeries were performed using standard aseptic techniques approved by NYU's IACUC review board. All mice were anesthetized with 3.5% isoflurane gas for ~two minutes, followed by ~1 hr (for mPFC-only surgeries) or ~2hrs (for mPFC and DS surgeries) of 0.5-1.5% continuous isoflurane throughout the surgery. The mPFC was targeted with coordinates of: AP: +2.34; ML: $\pm$ 0.4; DV: -2.15. The medial DS was targeted with coordinates of: AP: +0.5; ML: $\pm$ 1.5; DV: -3. Virus was delivered by the Drummond NanoJectII (Cat# 3-000-204; Broomall, PA), set to release 9.2nL of virus at a rate of 23nl/sec. This amount was released every 10s until the target volume was achieved. After injection, the pipette tip was left undisturbed at the target site for ~6 minutes.

Drug delivery. C21, the agonist of Gq-coupled DREADD, was given at a dosage of 1 mg/kg body weight (Tocris, Minneapolis, MN; Cat#5548). This dosage was achieved by first dissolving C21 in

DMSO for a 1mg/25µl stock solution and then further diluting by 1:400 with sterile saline. The Gi-coupled mutant kappa-opioid receptor (KORD) ligand, Salvinorin B (SalB, Cayman Chemical, Ann Arbor, MI; Cat#23582) was given at a dosage of 10mg/kg body weight, which was achieved by first dissolving SalB in DMSO for a 0.01 mg/µl stock solution, and then further diluting by 1:5 in sunflower oil (Rapanelli M et al. 2017). These dosages have been shown to not alter spontaneous locomotion in rats lacking DREADD expression (Marchant NJ *et al.* 2016; Thompson KJ *et al.* 2018).

Elevated Plus Maze.

EPM tests were conducted on ~P55 between 6:30am and 8:30am, following the first night of FR of ABA2 to measure anxiety-like behaviors (Fig. 1B). EPM duration was 10 min, and was performed as previously described (Wable GS *et al.* 2015). The time spent and the number of entries into the open arms were recorded and analyzed using the EthoVision tracking system (versions 11 and 13; Noldus Information Technology). General locomotion during the EPM test was used as an exclusion criteria: 4 subjects from one cohort with abnormal locomotion (greater or less than one standard deviation) were removed.

Brain tissue preparation. Animals were euthanized by transcardial perfusion, as per previously published protocols (Chowdhury TG *et al.* 2013). In brief, animals were anesthetized using urethane (i.p. 1000 – 1500 mg/kg), then transcardially perfused with 0.01M phosphate buffer saline (PBS)-heparin (10,000 units per 500 ml), then with 250 ml of 4% paraformaldehyde (EM Sciences) in 0.1M phosphate buffer (PB) at a flow rate of 25 ml/min. Brains were post-fixed in

the same paraformaldehyde perfusate for at least two days at room temperature and 3 days at 4^{o}C before sectioning coronally, using a vibrating microtome (VT1000 S, Leica Microsystems, Buffalo Grove, IL) at a thickness set to 50 µm, and stored at 4^{o}C in 0.01 M PBS containing 0.05% sodium azide.

Immunohistochemistry. Free-floating tissue was treated with 1% hydrogen peroxide in PBS for 30 min, blocked and then incubated for with primary antibody in 0.01M PB, made isotonic with 0.9% sodium chloride (PBS) plus 1% (wt:v) of bovine serum albumin to minimize non-specific labeling and 0.05% sodium azide to retard bacterial growth (PBS-BSA-azide). Sections incubated in primary antibody at room temperature with constant agitation for 48-72 hours. Visualization of virally expressed fluorophores was enhanced by immunofluorescence. Rat anti-mCherry, was used to detect mCherry, the reporter fluorophore for Gq-DREADDs (Thermofisher Cat# M11217; RRID: AB_2536611; dilution of 1:1000). Chicken anti-GFP (Aves labs, Davis, CA; Cat# GFP-1020; RRID: AB_10000240; dilution of 1:500) was used to detect mCitrine, the reporter fluorophore for KORD, as well as the eBFP reporter for the retrograde cre virus, the latter of which labelled cell nuclei. Cell activation/suppression by DREADDs was assessed by the presence vs absence of phospho-c-Fos-immunoreactivity (Cell Signaling Tech, Danvers, MA; Cat#5348S; Phospho-c-Fos (Ser32)(D82C12)XP Rabbit mAb; RRID: AB_10557109; dilution of 1:800). GABA-INs were detected by mouse anti-GAD67 (EMD Millipore Cat# MAB5406; RRID: AB_2278725; dilution of 1:800).

Immunofluorescent secondary antibodies were diluted 1:1 with glycerol and stored at -20^{o}C. Working dilutions were prepared the same day as use at 1:100 in PBS-BSA-azide. Rat-anti-mCherry was detected by Alexa Fluor® 594 AffiniPure Goat Anti-Rat (Jackson ImmunoResearch, West Grove, PA; Cat# 112-585-143). Chicken anti-GFP was detected by Alexa Fluor® 488 AffiniPure Goat Anti-Chicken (Jackson ImmunoResearch; Cat# 103-545-155). Rabbit-anti-phospho-c-Fos was detected by Alexa Fluor® 647 AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch; Cat# 111-545-155). Mouse anti-GAD was detected by DyLight™ 405-conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch; Cat# 111-475-166). After incubation in secondary antibodies overnight, tissue was washed in 0.01M PBS, mounted on gelatin-coated slides and coverslipped with Vectashield Hardset mounting medium (Vector laboratories, Burlingame, CA; Cat# H-1400). Immunofluorescent images were captured on the Leica Confocal SP8 with Leica's Application Suite 2.6.0, and analyzed with ImageJ (provided by the NIH).

Specificity of the immunolabeling procedures and lack of cross-reactivity in the quadruple labeling conditions were verified by determining that immunolabeling was absent when the primary antibody was omitted or when the secondary antibody was of an inappropriate species, (anti-mouse, anti-rabbit, and anti-chicken IgG for mCherry; anti-rat, anti-chicken, anti-rabbit IgG for c-Fos, etc). Specificity of mCherry and GFP immunoreactivity was further verified by the absence of immunolabeling within brains injected with cre-dependent DREADDs but without the injection of retrograde cre.

Cell counting for triple immunofluorescence was performed on a randomly-selected single hemisphere of mPFC tissue with anteroposterior position approximating Bregma +2.34. A

box was drawn within the PL region, encompassing at least 60 mCherry+ cells spanning layers 2-6, and all cells labeled for mCherry, GAD, and/or c-Fos were counted. To count c-Fos accurately across brains, optical density was sampled for all nuclei in the channel reporting c-Fos and a threshold drawn to ensure uniform thresholding across tissue.

Electron microscopic immunocytochemistry. A randomly-selected single hemisphere of mPFC tissue with anteroposterior position approximating Bregma +2.34 was selected for EM analysis. Tissue was freeze-thawed 8 times to promote penetration of immunoreagents into vibratome sections (Wouterlood FG and B Jorritsma-Byham 1993). The primary antibodies used were the same as those used for immunofluorescence, except for GAD immunocytochemistry that used rabbit anti-GAD 65/67 (1:400, Millipore Cat #AB1511, Lot #2718324). The secondary antibodies used to detect rat anti-mCherry antibody were goat anti-rat IgG conjugated to ultra-small colloidal gold and diluted 1:100 (Cat 25181 from EM Sciences, Hatfield, PA) and biotinylated goat anti-rat IgG, diluted 1:200 (Vector Labs, BA-9400, lot ZB01216). Secondary antibodies used to detect rabbit anti-GAD antibody were biotinylated goat anti-rabbit IgG, diluted 1:200 (BA-1000, lot ZA-0924 from Vector Labs). All antibody dilutions were prepared using PBS-BSA-azide, and were applied to free-floating tissue under constant agitation at room temperature overnight or longer. Specificity of the immunolabeling procedures and lack of cross-reactivity in the dual labeling conditions were verified by determining that immunolabeling was absent when the primary antibody was omitted or when the secondary antibody was of an inappropriate species (anti-rabbit IgG for mCherry; anti-rat IgG for GAD). Specificity of mCherry

immunoreactivity was further verified by the absence of immunolabeling within brains injected with cre-dependent DREADDs but without the injection of the retrograde Cre virus.

Following all steps of immunocytochemistry, membrane preservation was enhanced using 1% glutaraldehyde in PBS. Subsequently, reduced concentration of osmium tetroxide (0.1%, instead of 1% OsO_4) was used for tissue that underwent immunolabeling by SIG, so as to be able to detect HRP-DAB while minimizing loss of SIG labels due to oxidation. Membrane preservation and contrast was enhanced further using tannic acid, uranyl acetate (Cat #22400 from EM Sciences, Hatfield, PA) and iridium tetrabromide (Terzakis JA 1968; Lozsa A 1974; Phend KD et al. 1995). The tissue was embedded in EMBED-812 and examined using the JEOL 1200 XL (JEOL Ltd, Tokyo, Japan), equipped with Hamamatsu CCD camera from AMT (Boston, MA, USA) with and without counterstaining with Reynold's lead citrate. Images were analyzed using ImageJ software (Version 1.51; NIH). Images for analysis were captured at 20,000X-60,000X magnification, scanning the specimen at the tissue-EPON interface to ensure that imaged cells were at the surface of the tissue where there is maximal antibody penetration. Quantification of the extent of axo-somatic synapses formed upon pyramidal neurons in layer 5 of mPFC was performed as previously described (Chen et al, 2015), and as described in the Methods section of this publication.