Cocaine Inhibits Dopamine D2 Receptor Signaling via Sigma-1-D2 Receptor Heteromers

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Abstract

Under normal conditions the brain maintains a delicate balance between inputs of reward seeking controlled by neurons containing the D1-like family of dopamine receptors and inputs of aversion coming from neurons containing the D2-like family of dopamine receptors. Cocaine is able to subvert these balanced inputs by altering the cell signaling of these two pathways such that D1 reward seeking pathway dominates. Here, we provide an explanation at the cellular and biochemical level how cocaine may achieve this. Exploring the effect of cocaine on dopamine D2 receptors function, we present evidence of σ1 receptor molecular and functional interaction with dopamine D2 receptors. Using biophysical, biochemical, and cell biology approaches, we discovered that D2 receptors (the long isoform of the D2 receptor) can complex with σ1 receptors, a result that is specific to D2 receptors, as D3 and D4 receptors did not form heteromers. We demonstrate that the σ1-D2 receptor heteromers consist of higher order oligomers, are found in mouse striatum and that cocaine, by binding to σ1, D2 receptor heteromers, inhibits downstream signaling in both cultured cells and in mouse striatum. In contrast, in striatum from σ1 knockout animals these complexes are not found and this inhibition is not seen. Taken together, these data illuminate the mechanism by which the initial exposure to cocaine can inhibit signaling via D2 receptor containing neurons, destabilizing the delicate signaling balance influencing drug seeking that emanates from the D1 and D2 receptor containing neurons in the brain.

Introduction

The striatum is the main input structure of the basal ganglia and consists of subcortical structures involved in the processing of information related with the performance and learning of complex motor acts and motivational processes and is altered in conditions such as Parkinson’s, Huntington’s and in drug addiction [1]. GABAergic striatal efferent neurons constitute more than 95% of the striatal neuronal population [2]. There are two major subtypes of GABAergic striatal efferent neurons: GABAergic dynorphinergic neurons, which express the peptide dynorphin and dopamine D1 receptors and GABAergic enkephalinergic neurons, which express the peptide enkephalin and dopamine D2 receptors [3]. In the case of drug addiction, and specifically cocaine, the dopaminergic pathway plays a critical role in the pathology [4,5], specifically, the two populations of D1 and D2 containing neurons. These two pathways can control novelty seeking and reward-dependent learning as well as having opposite effects on motor activity [6]. Early studies performed in D1 receptor knockout mice showed the importance of dopamine D1 receptor in cocaine action as the activation of D1 receptors was an absolute requirement for the induction of the cellular and behavioral responses to cocaine [7]. In addition to opposing the locomotor effects of D1, D2 containing neurons also serve to oppose drug reinforcement [8]. In the context of cocaine it is known that the D2 is essential for cocaine’s effects [9] as D2 receptors are required to enhance the rewarding properties of cocaine [10]. In D2−/− mutant animals the release of dopamine evoked by cocaine injection is dramatically higher compared to WT animals, and an intact D2-mediated signaling is required to elicit the rewarding and reinforcing effects of cocaine [11]. At the mechanistic level it was shown there is a switch from D2 to a D1 mediated increase on GABAergic IPSC in cocaine treated rats [12], and in models of long-term cocaine treatment it has been shown that D1 increases and D2 levels decrease [13]. Finally, it has been shown that the activation of postsynaptic D2 on striatopallidal neurons can facilitate drug reinforcement via inhibition of these neurons [8]. All of these studies point to a balance between D1 and D2 in
controlling the motivational processes and reinforcement in drugs of abuse, and specifically cocaine.

The initial mechanistic steps of cocaine binding and its effects on these two striatal populations of neurons (D1, and D2 receptor containing neurons) are not well understood. What is known is cocaine is able to exert part of its behavioral and cellular effect by elevating dopamine levels in the striatum [14]. It achieves this by binding to and inhibiting the presynaptic dopamine transporter (DAT) [15]. Cocaine is a high-affinity inhibitor of DAT and upon binding to DAT, cocaine causes a rapid increase in extracellular dopamine levels. Although DAT inhibition is required for cocaine’s effects, it is not the only required mechanism of action per the effects of D1 and D2 receptors discussed above. In fact, Cocaine is able to modulate dopamine signaling, via both the D1 and D2 family of dopamine receptors, which when activated can lead to stimulation or inhibition of signaling pathways. This provokes the question, how does cocaine seemingly influence two different receptor pathways? One potential answer lies in the fact that cocaine does not seem to bind the dopamine receptors directly but can bind to a receptor heteromer made up of the D1-like receptor family member, D1 and the σ1-receptor [16]. Through this latter interaction, cocaine can potentiate D1 receptor-mediated adenylyl cyclase activation, induce ERK1/2 phosphorylation and counteract the MAPK activation induced by D1 receptor stimulation [16]. However, as discussed above, D2 also plays a role in the early effects of cocaine. Here we explore the initial molecular events after cocaine exposure on the dopamine receptor D2 like family and test the hypothesis that σ1 receptor may provide the link between cocaine and the D1 and D2 receptor signaling balance.

**Materials and Methods**

**Ethics Statement**

The study received the approval of the Catalan Ethical Committee for Animal Use (CEAA/DMAH 4049 and 5664) and all procedures were performed to minimize animal suffering.

**Fusion Proteins and Expression Vectors**

Sequences encoding amino acids residues 1–155 and 155–238 of YFP Venus protein, and amino acids residues 1–229 and 230–311 of RLuc proteins were subcloned in pcDNA3.1 vector to obtain the YFP Venus (vVenus), cVenus and RLuc. At clones (mRLuc, cRLuc) hemi-truncated proteins expressed in pcDNA3.1 vector. The human cDNA for the long isoform of dopamine D2 receptors (D2 receptors), adenosine A2A or σ1 receptors cloned in pcDNA3.1 were amplified without their stop codons using sense and antisense primers harboring unique restriction sites to clone D2 and σ1 receptors cloned in pcDNA3.1 without its stop codon using sense and antisense primers harboring unique restriction sites (or RLuc restriction site of an Rluc-expressing vector). The fragments were then subcloned to be in-frame with RLuc, EYFP or GFP into the EcoRI and BamHI sites (EcoRI and BamHI sites or EcoRI and Xhol sites for σ1 receptor). The fragments were then subcloned to be in-frame with RLuc, EYFP or GFP into the EcoRI and BamHI or Xhol restriction site of an Rhuc-expressing vector (pRhuc-N1, PerkinElmer, Wellesley, MA), an EYFP expressing vector (EYFP-N3; enhanced yellow variant of GFP; Clontech, Heidelberg, Germany) or an GFP2 expressing vector (GFP2-N2, Clontech) respectively, to give the plasmids that express D2 and σ1 receptors fused to either nVenus, cVenus, nRLuc8 or cRLuc8 on the C-terminal end of the receptor (D2-cVenus, D2-nVenus, D2-ωRLuc8, D2-nRLuc8, σ1-cVenus, σ1-nRLuc8 or σ1-cRLuc8, respectively). When analyzed by confocal microscopy, it was observed that all fusion proteins showed similar subcellular distribution than naive receptors (see results and results not shown). Fusion of RLuc and YFP to D2 or A2A receptors did not modify receptor function as previously determined by cAMP assays [17].

**Cell Culture and Chemical Reagents**

HEK-293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated Fetal Bovine Serum (FBS; all supplements were from Invitrogen, Paisley, Scotland, UK). CHO cell lines were maintained in ßMEM medium without nucleosides, containing 10% fetal calf serum, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamate (300 μg/mL). Cells were maintained at 37°C in an atmosphere of 5% CO2, and were passaged when they were 80–90% confluent, i.e., approximately twice a week. HEK-293T or CHO cells were transiently transfected with the corresponding cDNAs by PEI (PolyEthyleneImine, Sigma, St. Louis, MO, USA) method as previously described [18] for the corresponding siRNA by lipofectamine (Invitrogen™, Carlsbad, USA) method following the instructions of the supplier. siRNA that targets both human and rodent σ1 RNA and a scrambled control siRNA were purchased from Invitrogen (catalog HSS 145543). All ligands used are diagrammed in Figure S1. Cocaine-HCl was purchased from Spanish Agencia del Medicamento n°: 2003C00220. PD144418 and PER were purchased from Tocris, Bristol, UK. Quinpirole and raclopride were purchased from Sigma, St. Louis, MO, USA.

**Immunocytochemistry**

For immunocytochemistry, cells were fixed in 4% paraformaldehyde for 15 min and washed with PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Then, after permeabilization with buffer A containing 0.2% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin. After 1 h at room temperature, cells were labeled with the primary mouse monoclonal anti-Rhuc receptor antibody (1/200, Milipore, CA, USA) or mouse monoclonal anti-σ1 receptor antibody (1/200; Chemicon) for 1 h, washed, and stained with the secondary Cy3 donkey anti-mouse antibody (1/200, Jackson Immunoresearch Laboratories, West Grove, PA, USA). D2 receptors fused to YFP protein were detected by their fluorescence properties. Samples were rinsed and observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany).

**BRET and BRET with BiFC Assays**

HEK-293T cells growing in six-well plates were transiently co-transfected with a constant amount of cDNA encoding for the receptor fused to RLuc or nRLuc8 and cRLuc8 proteins and with increasingly amounts of cDNA corresponding to the receptor fused to YFP or nVenus and cVenus proteins (see figure legends). To quantify receptor-YFP expression or receptor-reconstituted YFP Venus expression, cells (20 μg protein) were distributed in 96-well microplates (black plates with a transparent bottom) and fluorescence was read in a Fluoro Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm reading. Receptor-fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing the BRET donor alone. For BRET or BRET with...
BiFC measurements, the equivalent of 20 µg of cell suspension were distributed in 96-well microplates (Corning 3600, white plates; Sigma) and 5 µM coelenterazine H (Molecular Probes, Eugene, OR) was added. After 1 minute for BRET or after 5 min for BRET with BiFC of adding coelenterazine H, the readings were collected using a Mithras LB 940 that allows the integration of the signals detected in the short-wavelength filter at 485 nm (440–500 nm) and the long-wavelength filter at 530 nm (510–590 nm). To quantify receptor-RLuc or receptor-reconstituted RLuc8 expression luminescence readings were also performed after 10 minutes of adding 5 µM coelenterazine H. Both fluorescence and luminescence of each sample were measured before every experiment to confirm similar donor expressions (about 150,000 luminescent units) while monitoring the increase acceptor expression (10,000–70,000 fluorescent units). The net BRET is defined as [(long-wavelength emission)/short-wavelength emission] – Cf where Cf corresponds to [(long-wavelength emission)/short-wavelength emission] for the donor construct expressed alone in the same experiment. BRET is expressed as mili BRET units, mBU (net BRET×1000).

**SRET Assays**

HEK-293T cells growing in six-well plates were transiently co-transfected with constant amounts of cDNAs encoding for both receptor fused to RLuc and GFP proteins and with increasingly amounts of cDNA corresponding to the receptor fused to YFP protein and SRET was determined as previously described using a Mithras LB 40 [19].

**Striatal Slices Preparation**

Brains from WT littermates and σ1 receptor KO CD1 albino Swiss male mice (8 weeks old, 25 g of weight) were generously provided by Laboratorios Esteve (Barcelona, Spain) [20]. Brains were rapidly removed from animals and striatal slices were obtained as previously indicated [16,21].

**Coimmunoprecipitation**

Striatal slices from WT littermates and σ1 receptor KO mice were treated with medium or with 150 µM cocaine for 30 min. The striatal tissue was disrupted with a Polytron homogenizer in 50 mM Tris-HCl buffer, pH 7.4, containing a protease inhibitor mixture (1/1000, Sigma). The cellular debris was removed by centrifugation at 13,000 g for 5 min at 4°C, and membranes were obtained by centrifugation at 105,000 g for 1 h at 4°C. Membranes were solubilized in ice-cold immunoprecipitation buffer (phosphate-buffered saline (PBS), pH 7.4, containing 1% (v/v) Nonidet P-40) and incubated for 30 min on ice before centrifugation at 105,000 g for 1 h at 4°C. The supernatant (1 mg/ml of protein) was processed for immunocomplex precipitation as described in the immunoprecipitation protocol using a Dynabeads® Protein G kit (Invitrogen) using goat anti-D2 receptor antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). As negative control anti-FLAG antibody (1:1000, Sigma) was used. Protein was quantified by the bicinchoninic acid method (Pierce) using bovine serum albumin dilutions as standards. Immunoprecipitates were separated on a denaturing 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were blocked for 90 min in 5% Bovine (1% fat) dry milk and PBS-Tween 20 (0.05% V/V). The following primary antibodies were incubated overnight at 4°C in 5% milk and PBS-Tween 20 (0.05% V/V): mouse anti-D2 receptor antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-σ1 receptor antibody B-5 (sc-137075) (1:800, Santa Cruz Biotechnology, Santa Cruz, CA) and, after washing three times for 10 min in PBS Tween-20 (0.05% V/V), membranes were incubated with the secondary antibody rabbit anti-mouse-HRP (1:20,000, Dako, Glostrup, Denmark) for 1 h at room temperature in 5% milk and PBS-Tween 20 (0.05% V/V). After three washes with PBS Tween-20 (0.05% V/V) and a final wash with PBS, bands were detected with the addition of SuperSignal West Pico Chemiluminescent Substrate (Pierce) and visualized with a LAS-3000 (Fujiﬁlm). Analysis of detected bands was performed by Image Gauge software (version 4.0) and Multi Gauge software (version 3.0).

**In Situ Proximity Ligation Assays (PLA)**

Striatal slices from WT and σ1 receptor KO mice treated or not with 150 µM cocaine for 30 min, were mounted on slide glass and heteroreceptors were detected using the Duolink II in situ PLA detection Kit (OLink; Bioscience, Uppsala, Sweden). Slices were thawed at 4°C, washed in 50 mM Tris-HCl, 0.9% NaCl pH 7.8 buffer (TBS), peroxidized with TBS containing 0.01% Triton X-100 for 10 min and successively washed with TBS. After 1 h incubation at 37°C with the blocking solution in a pre-heated humidity chamber, slices were incubated overnight in the antibody diluted medium with a mixture of equal amounts of the primary antibodies mouse anti-σ1 receptor antibody B-5 (sc-137075, 1:500, see above) and the guinea-pig anti-D2 receptor antibody (1:500 Sigma) which specificity for D2 receptors was previously demonstrated [21]. Slices were washed as indicated by the supplier and incubated for 2 h in a pre-heated humidity chamber at 37°C with PLA probes detecting mouse or guinea pig antibodies, Duolink II PLA probe anti-mouse plus and Duolink II PLA probe anti-guinea minus (prepared following the instructions of the supplier) diluted in the antibody diluted to a concentration of 1:5. After washing at room temperature, slices were incubated in a pre-heated humidity chamber for 30 min at 37°C, with the ligation solution (Duolink II Ligation stock 1:5 and Duolink II Ligate 1:40). Detection of the amplified probe was done with the Duolink II Detection Reagents Red Kit. After exhaustively washing at room temperature as indicated in the kit, slices were mounted using the mounting medium with DAPI. The samples were observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany). Images were opened and processed with Image J confocal.

**Immunohistochemistry**

Striatal slices from WT and σ1 receptor KO mice were thawed at 4°C, washed in TBS, permeobilized with TBS containing 0.1% Triton X-100 for 10 min and successively washed with TBS. Slices were rocked in Blocking reagent 1% (Roche, Sant Cugat del Vallès, Spain) for 1 h at 37°C in a humidified atmosphere and incubated overnight at 4°C in a humidified atmosphere with the primary antibodies: mouse anti-σ1 receptor antibody B-5 (sc-137075, 1:100, see above) or the guinea-pig anti-D2 receptor antibody (1:100 Frontier Institute, Ishikari, Hokkaido, Japan), in 0.1% TBS-Tween, 0.1% BSA-Acetylated (Aurion, Wageningen, The Netherlands), 7% SND. Slices were washed in TBS-Tween 0.05% and left for 2 h at room temperature in a humidified atmosphere with the corresponding secondary antibodies: goat anti-mouse (1:200, Alexa Fluor 488, Invitrogen) and goat anti-guinea pig (1:200, Alexa Fluor 488, Invitrogen) in the same medium. Then, the slices were washed in TBS-Tween 0.05%, followed by a single wash in TBS before mounting in Mowiol medium (Calbiochem, Merck, Darmstadt, Germany), covered with a glass and left to dry at 4°C for 24 h. The sections were observed and imaged in a Leica SP2 confocal microscope.
cAMP Determination

Non transfected or transiently transfected CHO cells (see figure legends) were treated for 10 min with the indicated concentrations of D2 receptor agonist quinpirole, 30 μM cocaine or 100 nM of the σ1 receptor agonist PRE-084 alone or in combination. cAMP production was determined using [3H]cAMP kit (Amersham Biosciences, Uppsala, Sweden) following the instructions from the manufacturer.

ERK 1/2 Phosphorylation Assays

WT and KO ice striatal slices were treated for the indicated time with the indicated concentrations of cocaine and/or D2 receptor ligands, frozen on dry ice and stored at −80°C. When ERK1/2 phosphorylation assays were performed in cell cultures, CHO cells (48 h after transfection) were cultured in serum-free medium for 16 h before the addition of the indicated concentration of cocaine or/and D2 receptor ligands for the indicated time. Both, cells and slices were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β-glycerophosphate, 1% Triton X-100, 20 μM phenyl-arsonic oxide, 0.4 mM NaVO4 and protease inhibitor cocktail) and ERK 1/2 phosphorylation was determined as indicated elsewhere [16,22].

CellKey Label-free Assays

The CellKey system provides a universal, label-free, cell-based assay platform that uses cellular dielectric spectroscopy (CDS) to measure endogenous and transfected receptor activation in real time in live cells [23]. Changes in the complex impedance (DZ or dz) of a cell monolayer in response to receptor stimulation were measured. Impedance (Z) is defined by the ratio of voltage to current as described by Ohm’s law (Z = V/I). CHO cell clones stably expressing D2 receptors were grown to confluence in a CellKey Standard 96 well microplate that contains electrodes at the bottom of each well. For untreated cells or for cells preincubated (overnight at 37°C) with PTx (10 ng/ml), medium was replaced by HBSS buffer (Gibco) supplemented with 20 mM HEPES 30 minutes prior to running the cell equilibration protocol. A baseline was recorded for 5 minutes and then cells were treated with increasing concentrations of the D2 receptor agonist quinpirole or cocaine alone or in combination and data was acquired for the following 10 minutes. To calculate the impedance, small voltages at 24 different measurement frequencies were applied to treated or non-treated cells. At low frequencies, extracellular currents (iec) that pass around individual cells in the layer were induced. At high frequencies, transcellular currents (ite) that penetrate the cellular membrane were induced and the ratio of the applied voltage to the measured current for each well is the impedance. The data shown refer to the maximum complex impedance induced extracellular currents (Z/ieq) response to the ligand addition.

Results

σ1 Receptors form Heteromers with Dopamine D2 Receptors but not with the Other D2-like Receptor Family Members

We first examined whether the receptors of the D2-like family could directly interact with σ1 receptors and thus be a target for cocaine binding. To do this we used the Bioluminescence Resonance Energy Transfer (BRET) technology in HEK-293T cells expressing a constant amount of D2 long isoform, D3 or D4 dopamine receptors fused to Renilla Lucifere (RLuc) and increasing amounts of σ1 receptors fused to Yellow Fluorescence Protein (YFP). Clear BRET saturation curves were obtained in cells expressing D2-RLuc receptors and increasing amounts of σ1-YFP receptors with a BRETmax of 55 ± 7 nBU and a BRET50 of 28 ± 6 (Fig. 1a). In contrast, in cells expressing D2-RLuc or D2-RLuc and σ1-YFP receptors a low and linear non-specific BRET signal was obtained thus confirming the specificity of the interaction between D2-RLuc and σ1-YFP receptors (Fig. 1b). As a further control, cells were cotransfected with σ1-YFP receptors and adenosine A2a-Rluc receptors and no specific BRET signal was obtained (Fig. 1a). These results indicate that σ1 receptors selectively interact with dopamine D2 receptors and not with the other members of the D2-like receptor family. The σ1 receptors are predominantly found in the endoplasmic reticulum membrane and the plasma membrane [24] with one hypothesis that it may be acting as a chaperone protein [25]. The expression of σ1 and D2 receptors at the plasma membrane level was explored by analyzing the co-localization of both receptors by confocal microscopy. HEK-293T cells were used in the assays since they constitutively express σ1 receptors, but not DAT [16]. As expected, a punctate σ1 receptor staining in naive (Fig. 1c left panels, top images) or cocaine-treated (Fig. 1c right panels, top images) HEK-293T cells was detected. After transfection of the cDNA corresponding to D2 receptors, a co-localization of σ1 receptor and D2 receptors was detected at the plasma membrane level in cells not treated with cocaine (Fig. 1c left panels, bottom images) or in cells treated with 30 μM cocaine for 30 min (Fig. 1c right panels, bottom images).

Higher Order Complex Formation between σ1 Receptors and Dopamine D2 Receptors

Recent crystal structures have demonstrated that homodimers of GPCRs are possible, a fact that has been confirmed for dopamine D2 receptors [26–30]. Considering that σ1 may act as a chaperone like molecule we investigated the possible formation of higher order receptor complexes between σ1 and D2 receptor homomers. To test this we first needed to know whether σ1 receptors could form dimers, something that had not been reported. First, we tested if σ1 receptors can form dimers by BRET experiments in HEK-293T cells expressing a constant amount of σ1-RLuc receptors and increasing amounts of σ1-YFP receptors. A positive and saturable BRET signal was obtained with a BRETmax of 165 ± 35 nBU and a BRET50 of 22 ± 12 (Fig. 2a) indicating that σ1-σ1 homodimers can exist and demonstrating, for the first time, the oligomerization of σ1 receptors. Next, we tested whether D2 receptor homomers could interact with σ1 receptors by a combined BRET and FRET assay termed Sequential Resonance Energy Transfer (SRET) [19]. This assay involves two sequential energy transfer events, one bioluminescent energy transfer between RLuc and a blue shifted GFP2 and a second fluorescent energy transfer event between excited GFP2 and YFP (see Fig. 2b top scheme). In HEK-293T cells expressing a constant amount of σ1-RLuc and D2-GFP2 receptors and increasing amounts of σ1-YFP receptors, a net SRET saturation curve was obtained with a SRETmax of 269 ± 33 SU and a SRET50 of 92 ± 24 (Fig. 2b). Cells expressing constant amounts of adenosine A2a-RLuc and A2a-GFP2 receptors and increasing amounts of σ1-YFP receptors provided very low and linear SRET, according to the lack of interaction between A2a receptors and σ1 receptors. These results demonstrate that σ1 receptors are able to form heteromers with D2-D2 receptor homomers. A net SRET saturation curve was also obtained using HEK-293T cells expressing constant amounts of σ1-RLuc and D2-GFP2 and increasing amounts of σ1-YFP (SRETmax: 140 ± 28 SU; SRET50: 9 ± 3) but not when D2-RLuc and D2-GFP2 receptors were
replaced by A2A-RLuc and A2A-GFP receptors (Fig. 2c). These results demonstrate that D2 receptors are able to form heteromers with σ1 receptors homomers. Finally, we tested for a higher order interaction of receptor heteromers constituted by σ1 and D2 receptor homomers (σ1-σ1-D2-D2). This was done using a modified BRET assay that involves a double complementation assay [30]. A diagram showing the BRET with luminescence/fluorescence complementation approach (BRET with BiFC assay; see Methods) is shown in Figure 2d (top panel). Briefly, one receptor fused to the N-terminal fragment (nRluc8) and another receptor fused to the C-terminal fragment (cRluc8) of the Rluc8 act as BRET donor after Rluc8 reconstitution by a close receptor-receptor interaction and one receptor fused to an YFP Venus N-terminal fragment (nVenus) and another receptor fused to the YFP Venus C-terminal fragment (cVenus), act as BRET acceptor after YFP Venus reconstitution by a close receptor-receptor interaction. Accordingly, cells were co-transfected with a constant amount of the two cDNAs corresponding to D2-nRluc8 and D2-cRluc8 (equal amounts of the two cDNAs) and with a constant amount of the two cDNAs corresponding to σ1-nVenus and σ1-cVenus (equal amounts of the two cDNAs). Specific BRET would only be possible if Rluc reconstituted by D2-nRluc8-D2-cRluc8 dimerization is close enough to YFP Venus reconstituted by σ1-nVenus-σ1-cVenus dimerization. Higher order heterotetramers were in fact observed as evidenced by a positive BRET signal (Fig. 2d). As negative controls, cells expressing only three fusion proteins and the fourth receptor not fused provided neither a significant fluorescent signal nor a positive BRET (Figure 2d).

Collectively these results indicate that σ1-D2 receptor heteromers seem to be constituted by the interaction of receptor homomers and the minimal structural unit is the σ1-σ1-D2-D2 receptor heterotetramer.

The Effect of σ1 Receptor Ligands on σ1-D2 Receptor Heterotetramer

It is known that cocaine can bind to σ1 [25,31,32]. We sought to measure the effect of cocaine binding to σ1 receptors on σ1-D2 receptor heteromers using BRET. We performed BRET experiments in HEK-293T cells expressing a constant amount of D2-RLuc receptors and increasing amounts of σ1-YFP receptors in the presence or in the absence of cocaine. The BRET saturation curve was reduced when cells were treated for 30 min with 30 μM of cocaine (BRET max: 35±6 mBU; BRET 50: 26±8) indicating that cocaine binding to σ1 receptors induces structural changes in the σ1-D2 receptor heteromer. The cells treated (10 min) with the σ1 agonist PRE084 (100 nM; BRET max: 40±8 mBU; BRET 50-
31 ± 6) but not with the antagonist PD144418 (1 μM; BRETmax: 48 ± 3 mBU; BRET50: 20 ± 5) also showed a decrease in the BRET saturation curves. Interestingly, the σ1 antagonist PD144418 is able to revert the effect induced by cocaine (BRETmax: 52 ± 9 mBU; BRET50: 31 ± 7 in the presence of cocaine and PD144418) (Fig. 3a). To know if structural changes in σ1-σ3 receptor homomers or in D2-D2 receptor homomers can account for the ligand-induced effect on σ1-D2 receptor heteromers, we performed BRET experiments first in cells expressing σ1-RLuc and σ1–YFP receptors as indicated in Fig. 2a. Cells were treated for 10 min with 100 nM of the agonist PRE084 or 1 μM of the antagonist PD144418 or for 30 min with 30 μM of cocaine alone.

Figure 2. Higher order complex formation between σ1 receptors and dopamine D2 receptors in living cells. In (a) BRET saturation experiments were performed with HEK-293T cells co-transfected with σ1-RLuc cDNA (0.2 μg) and increasing amounts of σ1-YFP cDNA (0.1 to 0.6 μg cDNA). A schematic representation of a BRET process is shown at top in which the receptor fused to RLuc acts as donor and the receptor fused to YFP acts as acceptor. In (b) and (c) SRET saturation experiments were performed with HEK-293T cells co-transfected with: (b) a constant amount of D2-RLuc (0.6 μg) and D2-GFP2 (1 μg) receptor cDNA (squares) or A2aRLuc (0.3 μg) and A2a-GFP2 (0.5 μg) receptor cDNA, as negative control (triangles), and increasing amounts of σ1-YFP receptor (0.2 to 1.5 μg cDNA), (c) a constant amount of σ1-Rluc (0.3 μg) and D2-GFP2 (1 μg) (triangles) or A2-GFP2 (0.5 μM) as negative control (squares) receptor cDNA and increasing amounts of σ1-YFP receptor cDNA (0.2 to 1.5 μg). The relative amount of acceptor is given as the ratio between the fluorescence of the acceptor minus the fluorescence detected in cells only expressing the donor, and the luciferase activity of the donor (YFP/Rluc). A schematic representation of a SRET process is shown at top images in which two sequential energy transfer events between RLuc and GFP2 (BRET process) and between GFP2 and YFP (FRET process) occurs. In (d) BRET with luminescence/fluorescence complementation approach was performed measuring BRET in cells co-transfected with 1 μg of the two cDNAs corresponding to D2-nRLuc8 and D2-cRLuc8 and with 1.5 μg of the two cDNAs corresponding to σ1-nVenus and σ1-cVenus (5). As negative controls, cells transfected with the same amount of cDNA corresponding to D2-nRLuc8, D2-cRLuc8, σ1-nVenus and cVenus (1), D2-nRLuc8, D2-cRLuc8, σ1-cVenus and nVenus (2), D2-nRLuc8, σ1-nVenus, σ1-cVenus and cRLuc8 (3), or D2-cRLuc8, σ1-nVenus, σ1-cVenus and nRLuc8 (4) did not display any significant luminescence or positive BRET. A schematic representation of a BRET with luminescence/fluorescence complementation approach is given at the top image in which one receptor fused to the N-terminal fragment (nRLuc8) and another receptor fused to the C-terminal fragment (cRLuc8) of the RLuc8 act as BRET donor after RLuc8 reconstitution by a close receptor-receptor interaction and one receptor fused to an YFP Venus N-terminal fragment (nVenus) and another receptor fused to the YFP Venus C-terminal fragment (cVenus), act as BRET acceptor after YFP Venus reconstitution by a close receptor-receptor interaction. BRET or SRET data are expressed as means ± S.D. of five to six different experiments grouped as a function of the amount of BRET or SRET acceptor.
or with 1 μM PD144418. As shown in Fig. 3b, no significant changes in BRET\textsubscript{max} or BRET\textsubscript{50} were observed. Then, changes in the BRET saturation curve obtained in cells expressing a constant amount of D\textsubscript{2}-RLuc receptors and increasing amounts of D\textsubscript{2}-YFP receptors (BRET\textsubscript{max}: 44 ± 3 mBU; BRET\textsubscript{50}: 12 ± 4) were analyzed. The BRET saturation curve changed in cells treated for 10 min with 100 nM of PRE084 (BRET\textsubscript{max}: 27 ± 5 mBU; BRET\textsubscript{50}: 11 ± 4) or 30 min with 30 μM of cocaine (BRET\textsubscript{max}: 29 ± 2 mBU; BRET\textsubscript{50}: 19 ± 5) but not in cells treated for 10 min with 1 μM of PD144418 (BRET\textsubscript{max}: 44 ± 3 mBU; BRET\textsubscript{50}: 9 ± 3). Again the antagonist, PD144418, was able to revert the effect induced by cocaine (BRET\textsubscript{max}: 43 ± 2 mBU; BRET\textsubscript{50}: 16 ± 3) in cells pre-treated with PD144418 and cocaine (Fig. 3c). These data suggest structural changes in the complex brought about by binding of either the σ\textsubscript{1} agonist PRE084 or cocaine. To test whether the effect of PRE084 or cocaine on D\textsubscript{2}-D\textsubscript{2} heteromers are due to the presence of σ\textsubscript{1} receptors, assays were performed in cells whose σ\textsubscript{1} receptor expression was knocked-down using an RNAi approach (Fig. 3d). When we transfected a specific small interfering RNA (siRNA), a robust silencing of σ\textsubscript{1} receptor expression was obtained (Fig. S2). The treatment with the specific siRNA completely abolished the effect of cocaine or PRE084 on the BRET saturation curve. The treatment with PD144418 or PD144418 and cocaine had no effect on these knocked-down cells (Fig. 3d). These results suggest that ligand binding to σ\textsubscript{1} receptors induces strong changes in the structure of the D\textsubscript{2}-D\textsubscript{2} receptor homomers in the σ\textsubscript{1}-D\textsubscript{2} receptor heteromers.

### Cocaine Binding to σ\textsubscript{1} Receptors Modulates the D\textsubscript{2} Receptor Signaling in Transfected Cells

The cocaine-induced modifications of the quaternary structure of D\textsubscript{2} receptor homodimers in the σ\textsubscript{1}-D\textsubscript{2} receptor heteromer described above suggest that cocaine can modulate the functionality of D\textsubscript{2} receptors. To study how cocaine affects D\textsubscript{2} receptor-mediated signaling, Chinese hamster ovary (CHO) cells were used as they provided a lower baseline of signaling for which to detect downstream changes and have been shown to constitutively express σ\textsubscript{1} receptors but not DAT [16]. The effect of cocaine on D\textsubscript{2} receptor agonist-induced, G protein-mediated signaling was measured using a label free assay that measures changes in cell impedance in response to stimulation. In CHO cells stably expressing D\textsubscript{2} receptors, increasing cocaine concentrations (10 nM to 100 μM) did not give any G protein-mediated signaling, neither G\textsubscript{i/o}, G\textsubscript{s} or G\textsubscript{q} (Fig. 4a) as compared to known control receptors (Fig. S3). The signaling obtained upon D\textsubscript{2} receptor activation with the agonist quinpirole (0.1 nM to 1 μM) showed a G\textsubscript{i} profile (increases in impedance) that was completely blocked when cells were treated with the G\textsubscript{i} specific pertussis toxin (PTx) (Fig. 4b). We observed a small but significant decrease in the G\textsubscript{i} activation induced by quinpirole when cells were pre-treated for 1 h with 30 μM cocaine (Fig. 4c). These results indicate that cocaine by itself is not able to induce a G protein-mediated signaling but can partially inhibit the ability of D\textsubscript{2} receptors to signal through G\textsubscript{i}. A downstream consequence of G\textsubscript{i} mediated signaling is the ability to decrease cAMP signaling. In addition to the label free experiments above we determined the levels of cAMP in CHO cells stably expressing D\textsubscript{2} receptors using forskolin and then measured whether cocaine was able to decrease the forskolin-induced cAMP formation. We found cocaine alone could not decrease the levels of cAMP after treatment with forskolin compared to the D\textsubscript{2} agonist quinpirole (Fig. 4d). However, cocaine significantly dampened the quinpirole-induced decreases of forskolin-mediated increases in cAMP levels (Fig. 4d). This effect was blocked when cells were transfected with siRNA against the σ\textsubscript{1} receptor (Fig. 4d), demonstrating that cocaine’s ability to counteract the action of quinpirole was mediated by σ\textsubscript{1} receptors. Similar results were obtained when instead of cocaine the σ\textsubscript{1} receptor agonist PRE084 was used (Fig. S4) reinforcing the concept that σ\textsubscript{1} receptor ligands induce a significant decrease in the ability of D\textsubscript{2} receptors to signal through G\textsubscript{i}.

Apart from G protein-mediated signaling, many GPCRs are able to signal in a G protein-independent way [33–37]. ERK 1/2 phosphorylation is one of the MAPK pathways that has been described to be activated in a G protein-independent and arrestin-dependent mechanism [36]. Several reports have highlighted the importance of ERK 1/2 activation in D\textsubscript{2} receptors containing neurons for the effects of cocaine [36–41]. We sought to understand how cocaine might influence σ\textsubscript{1}-D\textsubscript{2} receptor heteromer-mediated ERK 1/2 signaling. Varying concentrations of cocaine and varying the time of treatment did not lead to any significant change in ERK 1/2 phosphorylation in response to cocaine in cells not expressing D\textsubscript{2} receptors (Fig. S5). Importantly, cocaine per se dose-dependently (Fig. S6a) and time-dependently (Fig. S6b) activated ERK 1/2 phosphorylation in cells expressing D\textsubscript{2} receptors. This effect was mediated by σ\textsubscript{1} receptors since it was strongly diminished in cells transfected with the σ\textsubscript{1} receptors siRNA (Figs. S6a and S6b). The D\textsubscript{2} receptor agonist quinpirole was also dose-dependently (Fig. S6c) and time-dependently (Fig. S6d) able to activate ERK 1/2 phosphorylation but, as expected, this effect was not mediated by σ\textsubscript{1} receptors since it was not diminished in cells transfected with the σ\textsubscript{1} receptors siRNA (Figs. S6c and S6d). These results point out that σ\textsubscript{1} or D\textsubscript{2} receptor activation in the σ\textsubscript{1}-D\textsubscript{2} receptor heteromer induces ERK 1/2 phosphorylation. Thus, cocaine, like quinpirole, can act as an agonist at the MAPK activation level for the heteromer.

A property of some receptor heteromers is the ability of the antagonist of one receptor to block the function of the agonist of the partner receptor, a property defined as cross-antagonism [22,42]. In cells expressing D\textsubscript{2} receptors we looked for cross-antagonism among σ\textsubscript{1}-D\textsubscript{2} receptor heteromers. Indeed we found the cocaine-induced ERK 1/2 phosphorylation was counteracted not only by the σ\textsubscript{1} receptor antagonist PD144418 (1 μM) but also by the D\textsubscript{2} receptor antagonist raclopride (10 μM) (Fig. 5a). Analogously, the D\textsubscript{2} receptor agonist quinpirole-induced ERK 1/2 phosphorylation was blocked by raclopride but also by PD144410 (Fig. 5b). These data suggest that antagonist binding leads to structural changes within the receptor heteromer that block signaling through the partner receptor. By definition an antagonist cannot signal on its own, therefore this cross-antagonism can only derive from the direct protein-protein interactions established between the receptors in the σ\textsubscript{1}-D\textsubscript{2} receptor heteromer. This hypothesis is further supported by the fact that silencing cells of the σ\textsubscript{1} receptor led to a complete loss in this cross-antagonism. That is, the effect of PD144410 on quinpirole-induced ERK1/2 phosphorylation was not observed when cells were transfected with the siRNA for σ\textsubscript{1} receptors (Fig. 5b).

As mentioned above cocaine can inhibit DAT and increase the dopamine concentration in the striatum; so, in the presence of cocaine both receptors in the σ\textsubscript{1}-D\textsubscript{2} receptor heteromer could be activated. Therefore we asked, what happens to ERK 1/2 phosphorylation after co-activation of both receptors? Surprisingly, a negative cross-talk was detected. When cells expressing D\textsubscript{2} receptors were treated with both 1 μM quinpirole and 30 μM cocaine there was a decrease in ERK 1/2 phosphorylation compared to quinpirole alone (Fig. 5c). This difference was not seen if the cells were depleted of σ\textsubscript{1} receptors via siRNA (Fig. 5c).
1-D2 Receptor Heteromers are Found in the Brain Striatum

The BRET experiments and the signaling experiments are all suggestive of functional complexes that can lead to changes in D2 receptor function. However, all of these experiments were performed in transfected cells. To establish whether these complexes and their functional implications can be seen in tissue we obtained striatum from wild type (WT) and σ1 knockout (KO) mice. The striatum expresses D2 receptor containing neurons of the indirect motor pathway and is one of the key areas of the brain where cocaine imposes its effects. First we examined whether σ1-D2 receptor heteromers could be detected in native tissue. We performed Western blot experiments and found the expression of both receptors in the striatum of WT mice and the expression of D2 receptors but not σ1 receptors in the striatum of KO mice (Fig. 6a). Next we performed co-immunoprecipitation experiments and found the antibody against D2 receptor could indeed co-precipitate D2 receptors and σ1 receptor (Fig. 6a) in WT mice striatum treated or not with 150 μM cocaine. This co-precipitation was not observed when tissue from σ1 receptor KO animals was used (Fig. 6a). Although supportive of the BRET experiments above and highly suggestive of heteromers in striatum, we wanted to ensure that these complexes were not an artifact of the detergent solubilization. To test this we used the recently developed proximity ligation assay on slices of striatum from both WT and σ1 KO mice [42]. Using immunohistochemistry, we first checked the expression of σ1 receptors in WT animals but not in KO animals (Fig. S7) and the expression of D2 receptors in both WT and KO animals (Fig. S8). Next we performed the proximity ligation assay on striatal slices from WT animals. The slices were treated or not with 150 μM cocaine and as shown in Figure 6 (b and d) a red punctate fluorescent staining was observed, indicating both receptors are indeed in a complex in mice striatum in the presence or absence of cocaine. As a negative control we repeated this with only one of the two primary antibodies, and staining was not seen (Fig. S9). As expected, the...
red punctate fluorescent staining was not observed when the experiments were performed with striatal slices from σ1 KO mice (Fig. 6c and e). These data further support the existence of σ1-D2 receptor heteromers in the striatum.

**Cocaine Binding to σ1 Receptors Modulates the D2 Receptor Signaling in Mouse Brain Striatum**

The above data provide strong evidence of σ1-D2 receptor heteromers in vivo but they do not say anything about the function of these complexes. We decided to test whether the negative cross-talk seen in signaling in transfected cells could also be found in the striatum. Striatum slices from WT and KO mice were tested for the effects of cocaine on ERK 1/2 phosphorylation. In co-transfected cells a strong and significant effect of cocaine was observed at 15 μM (see Fig. 5), a striatal level of the drug reached after pharmacologically significant doses of cocaine [43]. To allow diffusion into the tissue a ten-fold higher cocaine concentration, 150 μM, was then used to see clear effects in slices of mouse striatum. Both the D2 receptor agonist quinpirole (1 μM) and cocaine (150 μM) induced ERK 1/2 phosphorylation in striatal slices from WT mice after 10 min activation (Fig. S10) or after 30 min activation (Fig. 7a). More interestingly, in striatal slices of WT mice, the co-activation with quinpirole and cocaine...
blocked ERK 1/2 phosphorylation (Figs. 7a and S10). Thus, the negative cross-talk between σ₁ and D₂ receptors on MAPK signaling detected in cotransfected cells was also observed in striatal samples from WT mice, meaning that the same biochemical fingerprint seen in transfected cells was also found in WT mice. When similar experiments were performed in striatal slices from mice lacking the σ₁ receptors, cocaine was unable to induce ERK 1/2 phosphorylation (Figs. 7a and S10) and quinpirole-induced ERK 1/2 phosphorylation was not modified by cocaine (Figs. 7a and S10). These results strongly support the existence of functional σ₁-D₂ receptor heteromers in the striatum and indicate that all detected cocaine effects are dependent on σ₁ receptors expression.

**Discussion**

The data presented in this paper lead to several major conclusions on the role σ₁ receptors play in modulating D₂ receptor upon cocaine exposure. First, D₂ receptors can form heteromers with σ₁ receptors, a result that is specific to D₂ receptors as the other members of the D₂-like family, D₃ and D₄ receptors, did not form heteromers. Second, these σ₁-D₂ receptor heteromers are found in mouse striatum and are functional. Third, σ₁-D₂ receptor heteromers consist of higher order oligomers with a minimal structure of σ₁-σ₁-D₂-D₂ receptor heterotetramers. Finally, cocaine, by binding to σ₁-D₂ receptor heteromers, inhibits downstream signaling in both cultured cells and in mouse striatum.

Cocaine intake elevates dopamine levels in the striatum, particularly in its more ventral part, the nucleus accumbens, which has been shown to be a preferential anatomical substrate for reward [44,45]. Cocaine exploits the dopaminergic system to elicit part of its behavioral and cellular effects [14]. Earlier studies have suggested that the presynaptic dopamine transporter (DAT) is the primary target for cocaine effects [46–49]. However, not all cocaine effects are mediated by a dopamine increase derived by the cocaine inhibition of DAT. Indeed, cocaine interacts with many proteins, and it is now well established that cocaine interacts with σ₁ receptors at physiologically relevant concentrations [50–55]. In fact, reducing brain σ₁ receptor levels with antisense oligonucleotides attenuates the convulsive and locomotor stimulant actions of cocaine [56,57] and antagonists for σ₁ receptors have also been shown to mitigate the actions of cocaine in animal models [50,58]. σ₁ receptors are highly expressed in the brain [24,59]. Within the caudate-putamen and nucleus accumbens (the dorsal and ventral parts of the striatum, respectively), brain regions that mediate the long-term effects of cocaine, it was demonstrated that repeated cocaine administration induces up-regulation of σ₁ receptors, a process mediated by dopamine D₁ receptors [60]. Indeed, we have demonstrated earlier the importance of the σ₁ and D₁ receptor interaction on the initial events upon cocaine exposure [16]. In addition, others have shown σ₁ can modulate signaling of a different GPCR family [61]. Through σ₁-D₁ receptor heteromers, cocaine robustly potentiated D₁ receptor-mediated adenylyl cyclase activation, providing a mechanism for D₁ receptor-mediated effects of cocaine [16]. In addition to DAT and D₁ receptors, our work here highlights the importance of σ₁ receptors. Our data suggest that it is σ₁ receptors that are able to

![Figure 5. Cocaine binding to σ₁ receptor modulates the ERK 1/2 signaling in transfected cells.](image-url)
Figure 6. Expression of σ₁-D₂ receptor heteromers in the striatum. In (a) co-immunoprecipitation experiments are shown. Striatal slices from WT and KO mice were untreated or treated with 150 μM cocaine for 30 min. From slices solubilized striatal membranes (top panel) and immunoprecipitates with anti-D₂ receptor antibody or anti-FLAG antibody as negative control (NC) (middle and bottom panels) were analyzed by SDS-PAGE and immunoblotted using mouse anti-D₂ receptor antibody or mouse anti-σ₁ receptor antibody. IP: immunoprecipitation; WB: western blotting; MW, molecular mass. In (b to e) Proximity Ligation Assay (PLA) was performed as indicated in Materials and Methods, using WT (b and d) or KO (c and e) mouse striatal slices not treated (b and c) or treated (d and e) with 150 μM cocaine for 30 min. σ₁-D₂ receptor heteromers were visualized as red spots around blue colored DAPI stained nucleus. Scale bar: 20 μm.

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directly modulate the normally balanced D1 and D2 pathways via receptor-receptor interactions. The cocaine effect on σ1-D2 receptor heteromer signaling is in contrast with the cocaine effect on σ1-D2 receptor heteromer signaling described by Navarro et al [16]. In the D1 receptor-mediated activation of cAMP production was significantly increased by cocaine binding to a σ1, protomer in the σ1-D1 receptor heteromers, resulting in a cocaine-induced increase in cAMP production. The results here described and those described by Navarro et al [16], point to the scenario that is shown in Figure 6b, where cocaine selectively leads to increased dopamine-induced signaling through the cAMP pathway in D1 receptor-containing neurons and to depressed dopamine-induced inhibition of cAMP formation in D2 receptor-containing neurons. Simultaneously, cocaine alters the levels of the initial ERK 1/2 phosphorylation signaling induced by dopamine in both D1 receptor and D2 receptor-containing neurons. These findings suggest that cocaine exposure leads to a deregulation of a normally balanced D1/D2 dopamine receptor signaling (Fig. 7b). The balance of D1 and D2 inputs is designed to avoid addictive behavior, thus its disruption would have long term consequences. The data presented here support a key role of σ1 receptors in destabilizing this balance by increasing the D1 receptor-mediated cAMP production and dampening the D2 receptor signaling in σ1-D2 receptor heteromers, pushing the balance of inputs towards the D1 containing, pro-reward and motivating pathway. Our data is supported by the results described by Durieux and colleagues where they found that striatal D2R neurons can limit both dopamine pathways [54]. These are in line with our observations that the initial effects of cocaine disrupt the D1/D2 pathways. In summary, the results described here along with the highlighted previous studies support a model where the initial exposure to cocaine affects differently the direct (D1 containing) and indirect (D2 containing) pathways via σ1 receptor heteromers which may significantly influence dopaminergic neurotransmission.

Supporting Information

Figure S1 Chemical structure of compounds used. a) cocaine, b) σ1 receptor agonist PRE084, c) σ1 receptor antagonist PD144418, d) D2 receptor agonist quinpirole, e) D2 receptor antagonist raclopride.

Figure S2 Effect of σ1 receptor siRNA transfection on σ1 receptor expression. Membranes from non-transfected HEK-293T cells (wt) or cells transfected with σ1 receptor siRNA (6.25 μg of oligonucleotides) or irrelevant oligonucleotides (oligo, 6.25 μg of oligonucleotides) were analyzed by SDS/PAGE and immunoblotted with the anti-σ1 receptor antibody. Values are mean ± SEM of three experiments. ***P<0.001 compared with non-transfected cells (one-way ANOVA followed by Bonferroni post hoc tests).

Figure S3 Control CellKey label-free assays. HEK-293T cells were stably transfected with the G1 protein-coupled adenosine A2a receptor (a), the G3 protein-coupled adenosine A1 receptor (b) or untransfected (c) in 96 well Cell-Key plates. Impedance changes were measured using addition of 10 nM CGS 21680 (A2a receptor agonist) in (a), 10 nM CPA (A1 receptor agonist) in (b) or 50 nM thrombin (the agonist for the endogenous G4 protein-couples thrombin receptors) in (c). Plot shapes are consistent with the expected results for the respective G-proteins.

Figure S4 σ1 receptor agonist modulates the D2 receptor-mediated cAMP decreases. cAMP production was determined in CHO cells stably expressing D2 receptors not transfected (black columns) or transfected (white columns) with siRNA corresponding to σ1 receptor (6.25 μg of oligonucleotides). Cells were stimulated with 5 μM forskolin in absence (100%) or presence of 1 μM quinpirole, 100 nM PRE084 alone or in combination. Percent of cAMP produced respect to forskolin treated cells.

Figure S5 Cocaine effect on ERK 1/2 phosphorylation in cells not expressing D2 receptors. CHO cells were incubated with increasing cocaine concentrations for 30 min (a) or with 30 μM cocaine for increasing time periods (b). ERK1/2 phosphorylation is represented as percentage over basal levels (100%, non-treated cells). Results are mean ± SEM of three to four independent experiments performed in duplicate.
Figure S6 Cocaine-induced σ₁-D₂ receptor heteromer-mediated ERK 1/2 phosphorylation in transfected cells. CHO cells transfected with D₂ receptor cDNA (1 μg, black bars) or cotransfected (white bars) with D₂ receptor cDNA and σ₁ receptor siRNA (6.25 μg of oligonucleotides) were incubated with increasing cocaine concentrations for 30 min (a), with 30 μM cocaine for increasing time periods (b), with increasing quinpirole concentrations for 10 min (c) or with 1 μM quinpirole for increasing time periods (d). ERK1/2 phosphorylation is represented as percentage over basal levels (100%). Results are mean ± SEM of four to six independent experiments performed in duplicate. In all samples in (c) and (d) and samples without siRNA transfection in (a) and (b), Bi factorial ANOVA showed a significant (p<0.01) effect of cocaine or quinpirole over basal, and Bonferroni post hoc tests showed a significant counteraction of cocaine effect by siRNA (p<0.05, **p<0.01 and ***p<0.005 compared with sample with the same treatment and with siRNA transfection).

(TIF)

Figure S7 Expression of σ₁ receptor in the striatum. WT (a) or σ₁ receptor KO (b) mouse striatal slices were processed for immunohistochemistry as indicated in Materials and Methods using an anti-σ₁ antibody. Cell nuclei were stained with DAPI (blue). Scale bar: 20 μm.

(TIF)

Figure S8 Expression of D₂ receptor in the striatum. WT (a) or σ₁ receptor KO (b) mouse striatal slices were processed for immunohistochemistry as indicated in Materials and Methods using an anti-D₂ antibody (green). Scale bar: 20 μm.

(TIF)

Figure S9 Negative controls for in situ proximity ligation assays. Negative controls for in situ proximity ligation assays (see Materials and Methods) were performed in WT mouse striatal slices incubated with only anti-σ₁ (a) or anti-D₂ (b) antibody as primary antibodies. Cell nuclei were stained with DAPI (blue). Scale bar: 20 μm.

(TIF)

Figure S10 Negative cross-talk between cocaine and the D₂ receptor agonist quinpirole on ERK 1/2 phosphorylation in mouse striatum. WT (black bars) and σ₁ receptor KO (white bars) mouse striatal slices were treated for 10 min with 1 μM quinpirole, with 150 μM cocaine or with both. Immuno-reactive bands from six slices obtained from five WT or five KO animals were quantified for each condition. Values represent mean ± SEM of percentage of phosphorylation relative to basal levels found in untreated slices. No significant differences were obtained between the basal levels of the wild-type and the KO mice. Bi factorial ANOVA showed a significant (**p<0.01, ***p<0.005) effect over basal. One-way ANOVA followed by Bonferroni post hoc tests showed a significant cocaine-mediated counteraction of quinpirole (ΔΔΔΔΔΔp<0.005).

(TIF)

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Author Contributions

Conceived and designed the experiments: GN VC SF EC CL PJM. Performed the experiments: GN EM JB DF JM AC VC DA. Analyzed the data: GN EM VC SF EC CL PJM. Contributed reagents/materials/analysis tools: RF EC. Wrote the paper: EC SF CL PJM.

References


