Cellular/Molecular

Cocaine Disrupts Histamine H_3 Receptor Modulation of Dopamine D_1 Receptor Signaling: σ_1-D_1-H_3 Receptor Complexes as Key Targets for Reducing Cocaine's Effects

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The general effects of cocaine are not well understood at the molecular level. What is known is that the dopamine D_1 receptor plays an important role. Here we show that a key mechanism may be cocaine's blockade of the histamine H_3 receptor-mediated inhibition of D_1 receptor function. This blockade requires the σ_1 receptor and occurs upon cocaine binding to σ_1-D_1-H_3 receptor complexes. The cocaine-mediated disruption leaves an uninhibited D_1 receptor that activates G_s, freely recruits β-arrestin, increases p-ERK 1/2 levels, and induces cell death when over activated. Using *in vitro* assays with transfected cells and in *ex vivo* experiments using both rats acutely treated or self-administered with cocaine along with mice depleted of σ_1 receptor, we show that blockade of σ_1 receptor by an antagonist restores the protective H_3 receptor-mediated brake on D_1 receptor signaling and prevents the cell death from elevated D_1 receptor signaling. These findings suggest that a combination therapy of σ_1R antagonists with H_3 receptor agonists could serve to reduce some effects of cocaine.

Introduction

The striatum is involved in the learning and elicitation of reward-related behaviors. GABAergic striatal medium sized spiny neurons (MSNs) comprise ~95% of striatal neurons and receive two main extrinsic inputs, cortical-limbic-thalamic glutamatergic and mesencephalic dopaminergic, converging in the dendritic spines of the MSNs (Ferré et al., 2007). There are two distinct MSNs, giving rise to two efferent pathways of the basal ganglia, the striatonigral MSN or direct pathway and the striatopallidal MSN or the indirect pathway. These neurons selectively express

dopamine D_1 (D_1R) and D_2 (D_2R) receptors, respectively. The initiation of movement leads to dual input of these two pathways (Cui et al., 2013). Despite the dual input, it is the balance of both types of MSNs that determines the final striatal output and the facilitation and inhibition of specific motor responses involved in reward-related behavior (Gerfen and Surmeier, 2011). Drugs of abuse are able to subvert these balanced inputs by altering the cell signaling of striatopallidal and striatonigral MSN. In the case of cocaine, it binds to and inhibits the dopamine transporter (DAT) producing a large increase in extracellular dopamine (Williams and Galli, 2006). This is associated with an increase in D_1R signaling while D_2R density is decreased, tipping the balance of signaling toward the direct pathway (Pascoli et al., 2012; Volkow et al., 2013). Our work suggests that the role of D_1R in cocaine's effects depends on the ability of σ_1 receptors (σ_1R) to bind and differentially modulate D_1R and D_2R in both MSNs (Navarro et al., 2010, 2013). Cocaine, especially in the form of crack, is associated with toxic consequences such as seizures and death. The biochemical alterations that follow the intake of cocaine are not well understood, but several studies indicate that D_1R is involved in cocaine's effects (Ritz and George, 1997; Aksenov et al., 2006; Lepsch et al., 2009).

In addition to glutamatergic and dopaminergic inputs, the striatum receives hypothalamic histaminergic input, which releases histamine from asynaptic varicosities (Takagi et al., 1986).

Histamine H$_3$ receptors (H$_3$R) are highly expressed presynaptically and postsynaptically in the striatal spine module (Ellenbroek, 2013; Panula and Nuutinen, 2013) and mostly localized postsynaptically in both types of MSN where they can control the D$_1$R signaling through the formation of D$_1$R-H$_3$R receptor heteromers (Moreno et al., 2011a; Ellenbroek, 2013; Panula and Nuutinen, 2013). This heteromer acts as a relay where activated H$_3$R can serve as a "molecular brake" for D$_1$R signaling. This effect is reached through a molecular protein-protein interaction between receptors in the heteromer. This is a common biochemical property of receptor heteromers, and it is defined as an intermolecular interaction by which the presence of one receptor, or the ligand binding to one receptor unit in the heteromer, changes positively or negatively the binding and/or the functional properties of another receptor unit in the heteromer (Ferré et al., 2009). These interprotomer interactions or cross-talk have been described for D$_1$R-H$_3$R heteromers upon heteromer coactivation with agonists(Ferrada et al., 2009). Thus, through a negative cross-talk between receptors, H$_3$R agonist decreases the D$_1$R agonist affinity and signaling. Some receptor heteromers, including D$_1$R-H$_3$R heteromers, have been found to display cross-antagonism, the ability of an antagonist of one receptor to also antagonize the signaling of the partner receptor (Ferrada et al., 2009; Moreno et al., 2011b; González et al., 2012). Cross-antagonism requires heteromer formation for any cross-receptor effects as antagonists do not signal on their own. Thus, cross-antagonism can be used as a fingerprint for identification of the presence of the heteromer (Ferré et al., 2009). In this frame here we explore a new physiological role for D$_1$R-H$_3$R heteromers with the idea of using the H$_3$R-mediated inhibitory effects of D$_1$R signaling to dampen cocaine's effects. We found that H$_3$R activation blocks D$_1$R agonist-mediated cell death in striatal organotypic cultures. We discovered a potentially key biochemical mechanism involved in the effects of cocaine. Cocaine strongly disrupts D$_1$R-H$_3$R heteromer function, including the neuroprotective effects of H$_3$R activation in the D$_1$R-H$_3$R heteromer. We thus propose the association of σ_1R antagonists with H$_3$ receptor agonists as a therapeutic approach for cocaine abuse.

Materials and Methods

Animals, cocaine acute treatment, and cocaine self-administration. All animal protocols were in accordance with the European Communities Council Directive 86/609/EEC and approved by the Ethics Committee for Human and Animal Research of the Universitat Autònoma de Barcelona. A maximal effort was made to minimize the number and suffering of animals. Male Sprague Dawley rats were obtained from the Animal Service, Universitat Autònoma de Barcelona, Bellaterra, Spain. To obtain rats self-administered with cocaine, our protocol exhaustively described previously (Hoffmann et al., 2012) was used. To obtain rats acutely treated with cocaine, rats (6–11 weeks old) were conditioned to handling during previous days to a single 15 mg/kg cocaine intraperitoneal injection (free base) dissolved in saline. As naive rats, 9- to 11-week-old rats ($\pm$350 g) were housed in a temperature (21 $\pm$ 1°C) and humidity-controlled (55 $\pm$ 10%) room with a 12 h light/dark cycle with food and water *ad libitum*. In all cases, 4% isoflurane (2-chloro-2- (difluoromethoxy)-1,1,1-trifluoro-ethane) anesthetized animals were killed by decapitation to obtain rat brains. Brains from wild-type (WT) littermates and σ_1R knock-out (KO) CD1 albino Swiss male mice (8 weeks old, 25 g) were generously provided by Laboratorios Esteve (Barcelona, Spain). The characterization of these mice has been previously described (Langa et al., 2003). Cocaine-HCl was purchased from Spanish Agencia del Medicamento #2003C00220.

Striatal slices preparation. Mouse or rat brains were rapidly removed and placed in ice-cold oxygenated (O$_2$/CO$_2$: 95%/5%) Krebs-HCO$_3^-$ buffer (124 mM NaCl, 4 mM KCl, 1.25 mM KH$_2$PO$_4$, 1.5 mM MgCl$_2$, 1.5 mM CaCl$_2$, 10 mM glucose and 26 mM NaHCO$_3$, pH 7.4). The brains were sliced perpendicularly to the long axis of the striatum at 4°C using a brain matrix (Zivic Instruments). Slices (500 μm thick) were kept at 4°C in Krebs-HCO$_3^-$ buffer during the dissection. Each striatal slice was transferred into an incubation tube containing 1 ml of ice-cold Krebs-HCO$_3^-$ buffer. The temperature was raised to 23°C and after 30 min, the media was replaced by 2 ml of fresh Krebs-HCO$_3^-$ buffer (23°C). The slices were incubated under constant oxygenation (O$_2$/CO$_2$: 95%/5%) at 30°C for 4–5 h in an Eppendorf Thermomixer (5 Prime). The media was replaced by 200 μl of fresh Krebs-HCO$_3^-$ buffer and incubated for 30 min before the addition of any agent. Slices were treated or not with the indicated ligand for the indicated time. After the indicated incubation period, the solution was discarded, and slices were frozen on dry ice and stored at −80°C.

Organotypic striatal slice cultures and cell death determination. Coronal striatal slices (500 μm thickness) from 7- to 8-week-old rats were obtained using a brain matrix and cultured in Neurobasal medium supplemented with 20% horse serum, 0.5% B27, 2 mM L-glutamine, 100 μg/ml sodium pyruvate, MEM Non-Essential Amino Acids Solution (1/100), and 100 U/ml penicillin/streptomycin (all supplements were from Invitrogen). After 24 h, culture medium was replaced by fresh medium containing no ligands or the indicated concentrations of the σ_1R ligands cocaine or PRE-084. After 1 h, medium, SCH 23390, imetit, or thioperamide were added at the indicated concentrations and incubated for an additional 1 h before the addition of D$_1$R agonist. Slices were maintained 48 h more in culture. When the σ_1R antagonist PD 144418 was used it was added 1 h before the addition of cocaine. After the total 72 h of culture, 10 μM propidium iodide (PI) was added and maintained at 37°C for 1 h, then slices were washed twice in cold-PBS and fixed with 4% paraformaldehyde for 1 h at 4°C. Slices were stained with Hoechst 1:1000 and total and PI-positive nuclei were quantified using Leica SP2 confocal microscope (20×; UV, 561 lasers) and the quantification program Image-based Tool for Counting Nuclei for ImageJ.

Cell culture, expression vectors, and transient transfection. Human neuroblastoma SK-N-MC cell clone expressing human D$_1$R and H$_3$R was developed and grown as previously described (Ferrada et al., 2009) Human embryonic kidney (HEK293T) cells were grown in DMEM supplemented with 2 mM L-glutamine, 100 μg/ml sodium pyruvate, 100 U/ml penicillin/streptomycin, MEM Non-Essential Amino Acids Solution (1/100), and 5% (v/v) heat-inactivated fetal bovine serum. For transfection, sequences encoding fusion proteins H$_3$R-Rluc, D$_1$R-Rluc, σ_1R-YFP, H$_3$R-YFP, D$_1$R-YFP, and σ_1R-GFP^2, which correspond with the human receptors fused to *Renilla luciferase* (Rluc) or to fluorescent proteins YFP or GFP^2 on the C-terminal end of the receptor, were obtained and characterized as previously described (Ferrada et al., 2009; Navarro et al., 2010, 2013). Cells were transiently transfected with the corresponding fusion protein cDNA by the ramified polyethylenimine (PEI; Sigma) method (Ferrada et al., 2009; Navarro et al., 2010, 2013) or by the lipofectamine (Invitrogen) method, in transfections where siRNA was present, following the instructions of the supplier. siRNA that targets both human and rodent σ_1R RNA and a scrambled control siRNA were purchased from Invitrogen (catalog #HSS 145543). To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad) using bovine serum albumin (BSA) dilutions as standards.

Lentivirus production and cell transduction. Silencing lentiviral vectors were produced by cotransfecting HEK293 cells with lentiviral silencing plasmids GIPZ Human SIGMAR1 shRNA (Clone Id:V3LHS_377499 or V3LHS_406026; Thermo Scientific) with packing plasmid psPAX2 and envelope coding plasmid pMD2.G (Addgene #12260 and #12259, respectively) using the calcium phosphate method. For production of control nonsilencing lentiviral particles the σ_1R silencing plasmids were substituted with GIPZ Non-silencing Lentiviral shRNA Control (RHS4346; Thermo Scientific). Infectious lentiviral particles were harvested at 48 h post-transfection, centrifuged 10 min at 3000 rpm to get rid of cell debris, and then filtered through 0.45 μm cellulose acetate filters. The titer of recombinant lentivirus was determined by serial dilution on HEK293T cells. For lentivirus transduction, SK-N-MC cells were subcultured to 50% confluence, cells were transduced with σ_1R-shRNA-expressing len-

tivirus obtained with plasmid V3LHS_377499 (LV sh) or control-shRNA-expressing lentivirus (LV control) at a multiplicity of infection of 10 in the presence of polybrene 5 μg/ml. Virus-containing supernatant was removed after 3 h. Puromycin was added to the culturing media at the final concentration of 1 μg/ml 2 d after infection. Five days after puromycin selection cells were transduced with the second σ_1R-shRNA-expressing lentivirus obtained with plasmid V3LHS_406026 to improve the level of silencing achieved. LV control-infected cells were reinfected with control-shRNA-expressing lentivirus. The second infection was performed as the first one. Cells were tested 72 h after the second transduction was performed.

RNA and real-time PCR. RNA was extracted using TRIzol Reagent (Molecular Research Center). cDNA was synthesized from 2 μg total RNA (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). The mRNAs of GAPDH and σ_1R were measured by real-time (RT)-PCR with 1 μL cDNA and power SYBER green PCR Master Mix (Applied Biosystems). Primer sequences are as follows: hGAPDH For: GGCTGGGGCTCATTTGCAGGG, hGAPDH Rev: TGACCTTGGC-CAGGG GTGCT, σ_1R For: TTCCAGCGCGAAGAGATAGC, and σ_1R Rev: CCGTGTACTA CCGTCTCCC. Thermal cycling conditions for amplification were set at 50°C for 30 min and 95°C for 10 min, respectively. PCR denaturing was set at 95°C for 15 s and annealing/extending at 60°C for 60 s for 40 cycles. mRNA levels normalized for GAPDH are expressed as fold change relative to control cells. The results were quantified with the comparative C_t method (known as the $2^{-\delta\delta Ct}$ method).

Bioluminescent resonance energy transfer assays. HEK293T cells were transiently cotransfected with a constant amount of cDNA encoding for the receptor fused to Rluc and with increasingly amounts of cDNA corresponding to the receptor fused to YFP (see figure legends). To quantify receptor-YFP expression, fluorescence of cells (20 μg protein) was read in a Fluoro Star Optima Fluorimeter (BMG Labtechnologies) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter at 400 nm reading. For bioluminescent resonance energy transfer (BRET) or BRET with BiLFC measurements, 5 μM coelenterazine H (Invitrogen) was added to the equivalent of 20 μg of cell suspension. After 1 min for BRET or after 5 min for BRET with BiLFC after 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 and the long-wavelength filter at 530 nm. To quantify receptor-Rluc expression, luminescence readings were also performed after 10 min of adding 5 μM coelenterazine H. 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. Data were fitted to a nonlinear regression equation, assuming a single phase saturation curve with GraphPad Prism software. BRET is expressed as mili BRET units (mBU; net BRET × 1000).

Sequential resonance energy transfer assays. For sequential resonance energy transfer (SRET) assays, HEK293T cells growing in six-well plates were transiently cotransfected with constant amounts of cDNAs encoding for both receptor fused to Rluc and GFP^2 proteins and with increasing amounts of cDNA corresponding to the receptor fused to YFP protein. In SRET, the oxidation of the Rluc substrate DeepBlueC by receptor-Rluc triggers receptor-GFP^2 excitation (BRET), which triggers a subsequent excitation of receptor-YFP (FRET). Emission of YFP after addition of DeepBlueC is only possible if the three fusion proteins are in close proximity (<10 nm), allowing sequential bioluminescent and fluorescent resonance energy transfer to occur. Cells were used 48 h post-transfection. Using aliquots of transfected cells (20 μg of protein), different determinations were performed in parallel. (1) Quantification of protein-YFP expression was performed as indicated in BRET experiments. (2) For quantification of protein-Rluc expression cells were distributed in 96-well microplates (Corning 3600, white plates with white bottom), and luminescence was determined 10 min after addition of 5 μM coelenterazine H in a Mithras LB 940 multimode reader. (3) For SRET, cells were distributed in 96-well microplates (Corning 3600, white plates with white bottom), and 5 μM DeepBlueC (Invitrogen) was added. The SRET signal was collected using a Mithras LB 940 reader with detection filters for short wavelength (410 nm) and long wavelength (530 nm).

Table 1. Cocaine blocks the positive allosteric interaction between D$_1$R and H$_3$R.

Competition experiment ([^{3}H]SCH vs SKF38393)	Parameters	
	K_{D1} (nM)	K_{D2} (nM)
Not treated rats in the absence of cocaine		
Control	31 ± 4	1100 ± 200
+RAMH	14 ± 1*	320 ± 10*
Not treated rats in the presence of cocaine		
Control	28 ± 2	1000 ± 100
+RAMH	30 ± 4	1100 ± 200
Cocaine acutely treated rats		
Control	28 ± 4	440 ± 60
+RAMH	19 ± 4	390 ± 40
Sham rats		
Control	26 ± 4	700 ± 100
+RAMH	3 ± 2**	130 ± 40**
Cocaine self-administered rats		
Control	33 ± 6	800 ± 200
+RAMH	37 ± 9	1900 ± 600

Competition curves of the D$_1$R antagonist [^{3}H]SCH 23390 (2 nM) binding versus increasing concentrations of the D$_1$R agonist SKF 38393 were performed in the absence or the presence of 2 nM of the H$_3$R agonist RAMH using striatal membranes from control rats treated or not with 30 μM cocaine for 30 min, rats acutely treated with cocaine, or cocaine self-administered rats. K_{D1} and K_{D2} are, respectively, the equilibrium dissociation constants of SKF 38393 binding to D$_1$R and were determined by fitting binding data to Equation 3. Parameters are mean ± SEM ($n = 3$). For each group, significant differences with respect to control were calculated by an unpaired Student's t test (*$p < 0.05$, **$p < 0.01$).

Analogous with BRET, net SRET is defined as [(long wavelength emission)/(short wavelength emission)] − cf., where cf. corresponds to long wavelength emission/short wavelength emission for cells expressing protein-Rluc and protein-GFP^2. Linear unmixing was done for SRET quantification, taking into account the spectral signature to separate the two fluorescence emission spectra (Zimmermann et al., 2002). SRET is expressed as mili SRET units, mSU (net SRET × 1000). Data were fitted as in BRET experiments.

Striatal membrane preparation and coimmunoprecipitation experiments. Mouse or rat striatal tissue was homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing a protease inhibitor cocktail (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 washed three more times in the same conditions and were used for ligand binding assays or were solubilized for coimmunoprecipitation experiments with a Dynabeads Protein G kit (Invitrogen) using rat anti-D$_1$ receptor antibody (1:1000; Sigma). As negative control anti-calnexin antibody (1:1000; BD Biosciences PharMingen) was used. Immunoprecipitates were separated on SDS-polyacrylamide gel and transferred onto PVDF membranes to perform Western blot assays as previously described (Navarro et al., 2010; Moreno et al., 2011a) using as primary antibodies guinea pig anti-D$_1$R antibody (1:1000; Frontier Institute), rabbit anti-H$_3$R antibody (1:1000; Alpha Diagnostic), or mouse anti-σ_1R antibody B-5 (sc-137075) (1:800; Santa Cruz Biotechnology).

In situ proximity ligation assays. Mouse or rat striatal slices were mounted on slide glass, thawed at 4°C, washed in 50 mM Tris-HCl, 0.9% NaCl, pH 7.8 buffer (TBS), permeabilized with TBS containing 0.01% Triton X-100 for 10 min, and successively washed with TBS. Heteromers were detected using the Duolink II *in situ* PLA detection Kit (OLink Bioscience) following the instructions of the supplier. A mixture of equal amounts of the primary antibodies–guinea pig anti-D$_1$R antibody (see above) and rabbit anti-H$_3$R antibody (see above) or mouse anti-σ_1R antibody B-5 (see above) and guinea pig anti-D$_1$R antibody or mouse anti-σ_1R antibody B-5 and rabbit anti-H$_3$R antibody–were used to detect D$_1$R-H$_3$R, σ_1R-D$_1$R and σ_1R-H$_3$R heteromers. respectively. Slices were mounted using the mounting medium with DAPI. The samples were observed in a Leica SP2 confocal microscope (Leica Microsystems). Images were opened and processed with ImageJ confocal.

Cell signaling. The cAMP concentration was determined by HTRF methodology using the LANCE Ultra cAMP kit (PerkinElmer) and a PHERAstar Flagship Microplate Reader (BMG Labtechnologies) following the instructions of the supplier. To determine ERK 1/2 phosphoryla-
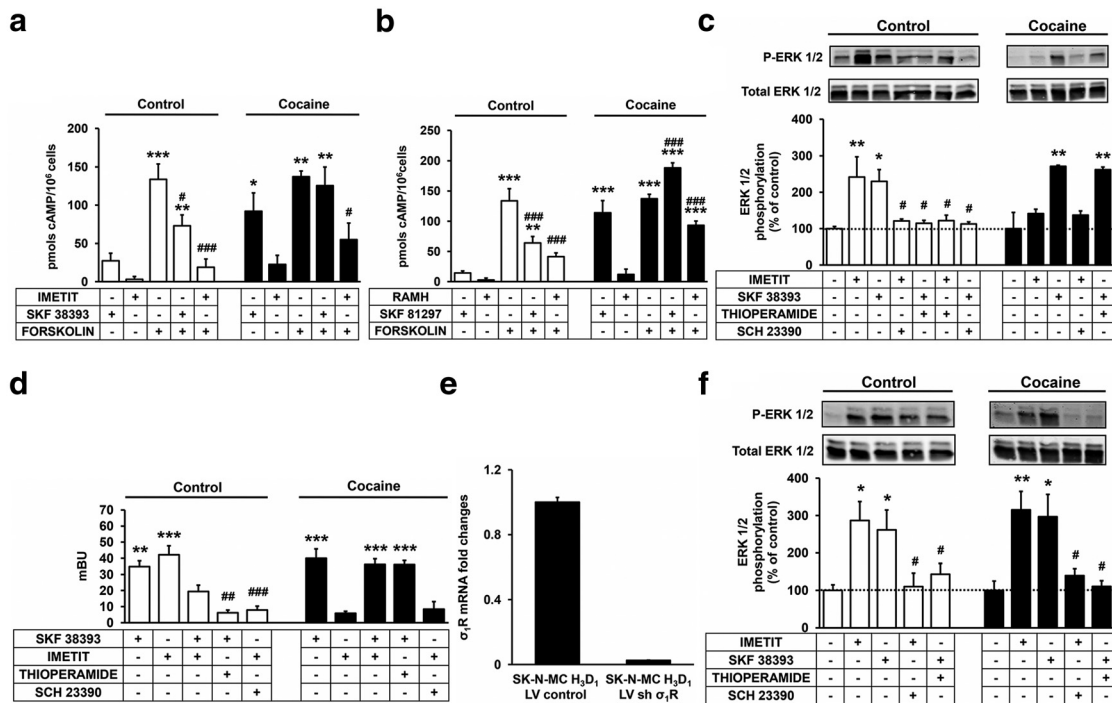
Figure 1. Cocaine alters the functional characteristics of D$_1$R-H$_3$R heteromers in transfected cells. *a–c, f*, Neuroblastoma SK-N-MC cells stable expressing D$_1$R and H$_3$R were not treated or treated overnight with 30 μM cocaine. *a, b*, cAMP production was determined after 10 min stimulation with D$_1$R agonist SKF 38393 (1 μM) or the H$_3$R agonist imetit (1 μM) (*a*) or with D$_1$R agonist SKF 81297 (100 nM) or the H$_3$R agonist RAMH (100 nM) (*b*) in the absence or in the presence of 0.5 μM forskolin. Values (cAMP produced in each condition minus basal stimulation in the absence of forskolin or agonists) represent mean $\pm$ SEM, $n = 4$–8. One-way ANOVA followed by Bonferroni *post hoc* test showed a significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) effect over basal (cells not stimulated) or (#$p < 0.05$, ###$p < 0.001$) over forskolin effect (cells stimulated with forskolin). *c, f*, Cells infected with the empty virus vector (*c*) or infected with lentiviral particles expressing shRNA (shσ_1R) to silence the receptor (*f*) were treated for 30 min with the D$_1$R antagonist SCH 23390 (1 μM) or the H$_3$R antagonist thioperamide (1 μM) before the addition of SKF 38393 (100 nM) or imetit (100 nM) for an additional incubation period of 10 min and ERK 1/2 phosphorylation was determined by Western blot. Values represent mean $\pm$ SEM ($n = 3$–5) of percentage of phosphorylation relative to basal levels found in untreated cells. One-way ANOVA followed by Bonferroni *post hoc* tests showed a significant (*$p < 0.05$, **$p < 0.01$) effect over basal or of the antagonist plus agonist treatment over the agonist treatment (#$p < 0.05$). *d*, β-Arrestin 1 recruitment was measured by BRET experiments in HEK293T cells 48 h post-transfection with 2 μg of cDNA corresponding to D$_1$R, 1 μg of cDNA corresponding to β-arrestin 1-Rluc, and 3 μg of cDNA corresponding to H$_3$R-YFP. Cells were not treated (BRET < 10) or treated for 30 min with 30 μM cocaine, 1 μM SCH 23390, or 1 μM thioperamide alone or in combination before 10 min stimulation with SKF 38393 (100 nM) or imetit (100 nM). Values represent mean $\pm$ SEM, $n = 4$. One-way ANOVA followed by Bonferroni *post hoc* tests showed a significant effect over basal (**$p < 0.01$, ***$p < 0.001$) or of the antagonist plus agonist treatment over the agonist treatment (##$p < 0.01$, ###$p < 0.001$). *e*, σ_1R expression was determined after silencing of σ_1R by shRNA. Neuroblastoma SK-N-MC cells stable expressing D$_1$R and H$_3$R were infected with lentiviral particles containing either control (LV control) or shRNA specific for σ_1R (LV sh σ_1R) and the relative expression levels were tested using quantitative RT-PCR. Results are expressed as an average of three replicates.

tion, cells and mouse or rat striatal slices were treated or not with the indicated ligand for the indicated time and were lysed by the addition of 500 μl of 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 phenylarsine oxide, 0.4 mM NaVO$_4$, and protease inhibitor cocktail). The cellular debris was removed by centrifugation at 13,000 $\times$ *g* for 5 min at 4°C and the protein was quantified by the bicinchoninic acid method using BSA dilutions as standard. ERK1/2 phosphorylation were then detected as described previously (Moreno et al., 2011a). Arrestin recruitment was determined by BRET experiments (see above) in HEK293T cells 48 h after transfection with the indicated amounts of cDNA corresponding to D$_1$R, β-arrestin 1-Rluc, and H$_3$R-YFP. Cells (20 μg of cell suspension protein per well in a 96-well microplates) were not treated or treated for 30 min with 30 μM cocaine or 1 μM of the antagonists SCH 23390 or thioperamide alone or in combination, and 5 μM coelenterazine H was added before stimulation with the agonists SKF 38393 (100 nM) or imetit (100 nM) for 12 min and BRET between β-arrestin 1-Rluc and H$_3$R-YFP was determined as above.

Radioligand binding experiments and binding data analysis. Ligand binding was performed with rat striatal membranes (0.2 mg of protein/ml) in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl$_2$, at 25°C. For competition experiments, membranes were incubated with free 2 nM [^{3}H]SCH 23390 (PerkinElmer) or free 3 nM [^{3}H]R-a-methyl histamine ([^{3}H]RAMH; GE Healthcare) or free 2 nM [^{3}H] YM-09151-02 (PerkinElmer) in the presence or the absence of increasing free concentrations of SKF 38393 (0.01 nM–30 μM; Tocris

Bioscience) with or without 2 nM RAMH (Sigma), increasing free concentrations of SCH 23390 (0.01 nM–10 μM; Sigma), increasing free concentrations of RAMH (0.01 nM–30 μM), or increasing free concentrations of PRE-084 (0.01 nM to 10 μM; Tocris Bioscience). In all cases, membranes were incubated with ligands providing enough time to achieve stable equilibrium for the lower ligand concentrations. Nonspecific binding was determined in the presence of 30 μM nonlabeled ligand and confirmed that the value was the same as calculated by extrapolation of the competition curves. Free and membrane bound ligands were separated by rapid filtration of 500 μl aliquots in a cell harvester (Brandel) through Whatman GF/C filters embedded in 0.3% PEI that were subsequently washed for 5 s with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated overnight with 10 ml of Ecoscint H scintillation mixture (National Diagnostics) at room temperature and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer) with an efficiency of 62%. Protein was quantified by the bicinchoninic acid method (Pierce Chemical) using BSA dilutions as standard.

Competition curves were analyzed by nonlinear regression, using the commercial GraFit software (Erithacus software), by fitting the binding data to the two-state dimer receptor model (Franco et al., 2005, 2006) To calculate the macroscopic equilibrium dissociation constants Equation 1 deduced by Casadó et al. (2007) was used.

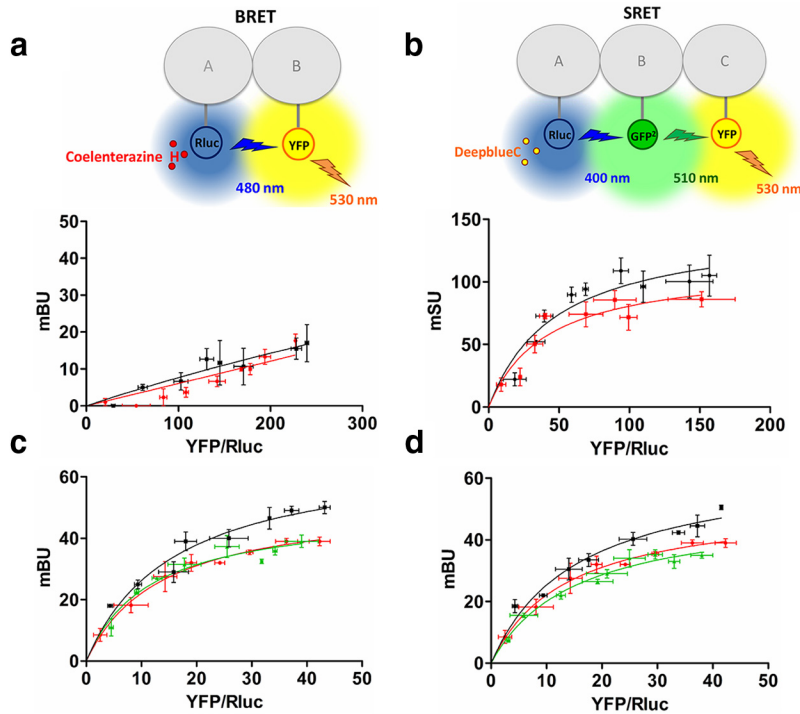$$A_{bound} = (K_{DA2}A + 2A^2 + K_{DA2}AB/K_{DAB})R_T \ (K_{DA1}K_{DA2}$$

Figure 2. σ1R-D1R-H3R complexes detected by energy transfer experiments. *a, c, d*, BRET saturation experiments were performed using HEK293T cells 48 h post-transfection with 0.2 μg of cDNA corresponding to H3R-Rluc (*a*) or 0.15 μg of cDNA corresponding to D1R-Rluc (*c, d*) and increasing amounts of cDNA corresponding to σ1R-YFP (*a*; 0.1–0.5 μg cDNA) or H3R-YFP (*c, d*; 0.2–1.5 μg cDNA). Cells were treated for 30 min (*a, c*) or 24 h (*d*) with medium (red lines) or with 30 μM cocaine (black lines) prior BRET determination. *c, d*, Cells were also transfected with siRNA for σ1R (see Materials and Methods; green line). Both fluorescence and luminescence of each sample were measured before every experiment to confirm similar donor expressions (~110,000 bioluminescence units) while monitoring the increase in acceptor expression (10,000 – 40,000 fluorescence units). *b*, SRET saturation experiments were performed using HEK293T cells 48 h post-transfection with 0.3 μg of cDNA corresponding to H3R-Rluc, 0.23 μg of cDNA corresponding to σ1R-GFP², and increasing amounts of cDNA corresponding to D1R-YFP (0.05–0.6 μg). Cells were treated for 30 min with medium (red lines) or with 30 μM cocaine (black lines) prior to SRET determination. The relative amount of BRET or SRET is given as a function of 100 × the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (Rluc). BRET and SRET are expressed as mBU or mSU, respectively, and values are means ± SEM; three to four different experiments grouped as a function of the amount of acceptor. *a, b*, Top, A schematic representation of the BRET (*a*) and SRET (*b*) techniques is shown.

$$+ K_{DA2}A + A^2 + K_{DA2}AB/K_{DAB} + K_{DA1}K_{DA2}B/K_{DB1}$$

$$+ K_{DA1}K_{DA2}B^2/(K_{DB1}K_{DB2})), \quad (1)$$

where A represents the radioligand concentration, R_T is the total amount of receptor dimers, and K_{DA1} and K_{DA2} are the macroscopic dissociation constants describing the binding of the first and the second radioligand molecule (A) to the dimeric receptor; B represents the assayed competing compound concentration, and K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B. K_{DAB} can be described as a hybrid equilibrium radioligand/competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semi-occupied by A.

To quantify cooperativity, the dimer cooperativity index for the competing ligand B is defined by Casadó et al. (2007) as follows:

$$D_{CB} = \log (4K_{DB1}/K_{DB2}). \quad (2)$$

Depending on the characteristics of the ligands (the radioligand A and the competitor B) the following simplifications of the Equation 1 were considered:

For A ([³H]SCH 23390) noncooperative and B (SKF 38393) cooperative (biphasic competition curves; Table 1): Equation 1 can be simplified to Equation 3 due to the fact that $K_{DA2} = 4K_{DA1}$ and, therefore, K_{DA1} is enough to characterize the binding of the radioligand A (Casadó et al., 2007):

$$A_{bound} = (4K_{DA1}A + 2A^2$$

$$+ 4K_{DA1}AB/K_{DAB})R_T/(K_{DA1}^2 + 4K_{DA1}A$$

$$+ A^2 + 4K_{DA1}AB/K_{DAB} + 4K_{DA1}^2B/K_{DB1}$$

$$+ 4K_{DA1}^2B^2/(K_{DB1}K_{DB2})). \quad (3)$$

For A ([³H] YM-09151–02) and B (B_{max} determination for σ1R in Fig. 7): Equation 1 was simplified to Equation 4 due to the fact that $K_{DA2} = 4K_{DA1}$ and $K_{DB2} = 4K_{DB1}$ (Casadó et al., 2007):

$$A_{bound} = (4K_{DA1}A + 2A^2$$

$$+ 4K_{DA1}AB/K_{DAB})R_T/(4K_{DA1}^2 + 4K_{DA1}A$$

$$+ A^2 + 4K_{DA1}AB/K_{DAB} + 4K_{DA1}^2B/K_{DB1}$$

$$+ 4K_{DA1}^2B^2/K_{DB1}^2). \quad (4)$$

For A ([³H]SCH 23390 or [³H]RAMH) and B (SCH 23390 or RAMH) noncooperative being the A and B the same compound (B_{max} determination for D1R or H3R in Fig. 7): Equation 1 was simplified to Equation 5 due to the fact that $K_{DA2} = 4K_{DA1}$, $K_{DB2} = 4K_{DB1}$, $K_{DA1} = K_{DB1}$, $K_{DA2} = K_{DB2}$ and $K_{DAB} = 4K_{DA1}$ (Casadó et al., 2007):

$$A_{bound} = (4K_{DA1}A + 2A^2AB)$$

$$R_T/(4K_{DA1}^2 + 4K_{DA1}A + A^2$$

$$+ AB + 4K_{DA1}B + B_2). \quad (5)$$

Goodness of fit was tested according to reduced χ^2 value given by the nonlinear regression program.

Results

D1R-H3R heteromers are signaling units that can be modulated by cocaine

It has previously been described that the formation of D1R-H3R heteromers in neuroblastoma cells leads to a change in the D1R G-protein coupling from the G$_s$ to the G$_i$ protein, to which H3R is coupled. In fact, in the presence of the H3R, D1R was no longer coupled to G$_s$, and could not activate adenylyl cyclase, yet the heteromer remains coupled to G$_i$ (Ferrada et al., 2009). This change in G-protein coupling was identified as a specific characteristic of the heteromer. We first examined whether cocaine is able to influence the G-protein coupling to D1R-H3R heteromers by measuring cAMP production, which is tied to the activation of G-proteins. In SK-N-MC neuroblastoma cells expressing D1R and H3R, the D1R agonist SKF 38393 was able to reduce the forskolin-stimulated cAMP production in accordance with a G$_i$ protein coupling (Fig. 1a). Interestingly, in the presence of 30 μM cocaine, SKF 38393 increases cAMP production and did not affect forskolin-induced cAMP, in agreement with a G$_s$ coupling (Fig. 1a). In contrast with D1R agonist, the H3R agonist imetit induced a decrease in cAMP production in the presence or in the absence of cocaine in accordance with a G$_i$-protein coupling (Fig. 1a). Similar results were obtained using the H3R agonist RAMH and the D1R agonist SKF 81297 (Fig. 1b). These data suggest that cocaine may alter the G-proteins associated with the heteromer or at a minimum alter D1R-H3R signaling. Next, we examined whether cocaine could
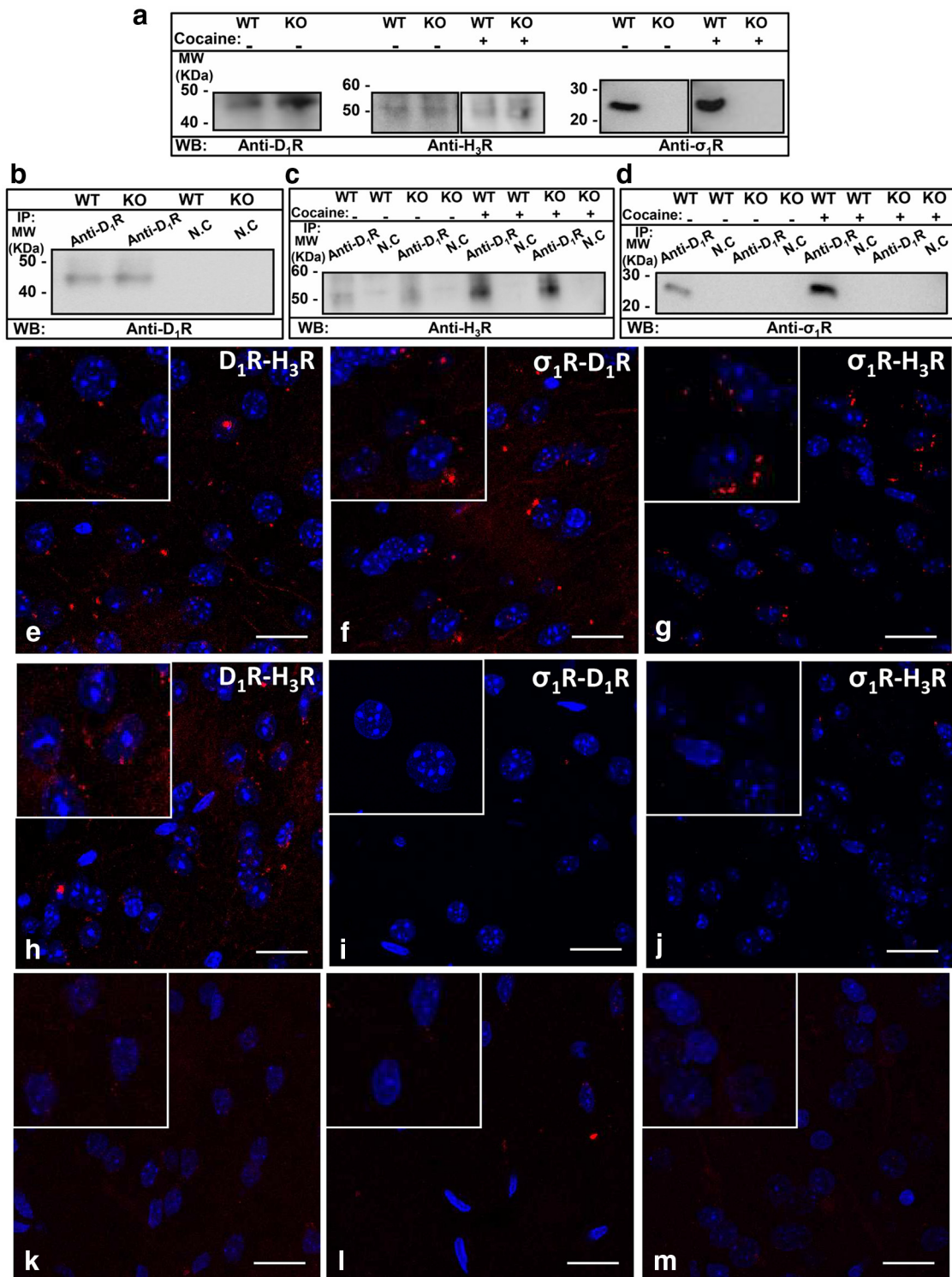
Figure 3. Expression of σ_1R-D_1R-H_3R complexes in the mouse striatum detected by coimmunoprecipitation and PLA experiments. **a–d**, Coimmunoprecipitation experiments are shown. Striatal slices from WT and σ_1R KO mice were untreated or treated with 150 μM cocaine for 30 min (cocaine). Solubilized striatal membranes (**a**) and immunoprecipitates with anti-D_1R antibody or anti-calnexin antibody as negative control (NC; **b–d**) were analyzed by SDS-PAGE and immunoblotted using anti-D_1R antibody (**b**), anti-H_3R antibody (**c**), or anti-σ_1R antibody (**c**). IP, immunoprecipitation; WB, Western blotting; MW, molecular mass. **e–m**, PLAs were performed as indicated in Materials and Methods, using WT (**e–g**) or σ_1R KO mice (**h–j**) striatal slices. D_1R-H_3R (**e, h**), σ_1R-D_1R (**f, i**), and σ_1R-H_3R (**g, j**) heteromers were visualized as red spots around blue-colored DAPI-stained nucleus. **k–m**, Negative controls were performed doing the PLA experiments in WT mouse striatal slices incubated with only anti D_1R (**k**), anti-σ_1R (**l**), or anti-H_3R (**m**) antibody as primary antibodies. Cell nuclei were stained with DAPI (blue). For each part a magnification is shown. Scale bar, 20 μm.

disrupt the cross-antagonism between both receptors in the heteromer previously described (Moreno et al., 2011a). In SK-N-MC neuroblastoma cells expressing D_1R and H_3R, the D_1R agonist SKF 38393 induced ERK 1/2 phosphorylation that was similar in the absence or the presence of cocaine suggesting that this signal was G-protein independent. SKF 38393-induced ERK 1/2 phosphorylation was blocked by its own antagonist SCH 23390 as well as by the H_3R antagonist thioperamide, but this
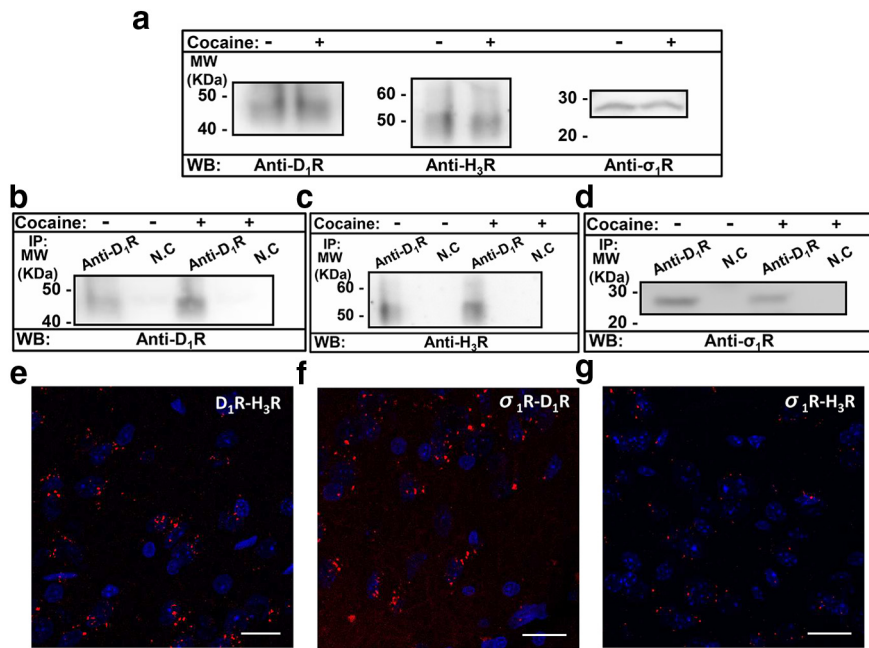
Figure 4. Expression of σ_1R-D$_1$R-H$_3$R complexes in the rat striatum detected by coimmunoprecipitation and PLA experiments. *a–d*, Coimmunoprecipitation experiments are shown. Rat striatal slices were untreated or treated with 150 μM cocaine for 30 min (cocaine). Solubilized striatal membranes (*a*) and immunoprecipitates with anti-D$_1$R antibody or anti-calnexin antibody as negative control (NC; *b–d*) were analyzed by SDS-PAGE and immunoblotted using anti-D$_1$R antibody (*b*), anti-H$_3$R antibody (*c*), or anti-σ_1R antibody (*c*). IP, immunoprecipitation; WB, Western blotting; MW, molecular mass. *e–g*, PLAs were performed as indicated in Materials and Methods, using rat striatal slices. D$_1$R-H$_3$R (*e*), σ_1R-D$_1$R (*f*), and σ_1R-H$_3$R (*g*) heteromers were visualized as red spots around blue–colored DAPI-stained nucleus. Scale bar, 20 μm.

Figure 5. Cocaine inhibits the H$_3$R signaling and the D$_1$R-H$_3$R cross-antagonism in ERK 1/2 phosphorylation in mouse striatum. WT (*a*) and σ_1R KO (*b*) mouse striatal slices were treated or not with 150 μM cocaine for 2 h and were preincubated for 20 min with medium, the D$_1$R antagonist SCH 23390 (10 μM), or the H$_3$R antagonist thioperamide (10 μM) before the addition of the D$_1$R agonist SKF 38393 (1 μM) or the H$_3$R agonist imetit (1 μM) for an additional incubation period of 10 min. ERK 1/2 phosphorylation was determined by Western blot. Immunoreactive bands from three to eight slices obtained from eight WT or KO animals were quantified for each condition. Values represent mean ± SEM percentage of phosphorylation relative to basal levels found in untreated slices. No significant differences were obtained between the basal levels of the WT and the σ_1R KO mice. One-way ANOVA followed by Bonferroni *post hoc* tests showed a significant (*$p < 0.05$) effect over basal or of the antagonist plus agonist treatment over the agonist treatment (#$p < 0.05$, ##$p < 0.01$).

Apart from G-protein-mediated signaling, many GPCRs are able to signal by an arrestin recruitment-dependent mechanism with p-ERK 1/2 being one of the signaling pathways downstream of arrestin (Shenoy and Lefkowitz, 2003; Shenoy et al., 2006; DeWire et al., 2007). If cocaine is able to alter the D$_1$R-H$_3$R heteromer-driven activation of ERK 1/2 then one possibility is that it also alters arrestin recruitment. Using BRET to measure β-arrestin 1 recruitment we investigated if cocaine is able to modulate the ligand-induced β-arrestin 1 recruitment to these receptors. In HEK293 cells transiently expressing D$_1$R, β-arrestin 1-Rluc, and H$_3$R-YFP, a positive BRET signal between β-arrestin 1 and H$_3$R was detected after stimulation with H$_3$R agonist imetit or with D$_1$R agonist SKF 38393 (Fig. 1*d*). Interestingly, the BRET signal was diminished when cells were coactivated with both agonists, indicating a negative cross-talk between both receptors and in line with that seen in neurons (Arias-Montaño et al., 2001). In addition, we could also detect cross-antagonism in the β-arrestin 1 recruitment assays, that is, the BRET induced by SKF 38393 was blocked by the H$_3$R antagonist thioperamide and the BRET induced by imetit was blocked by the D$_1$R antagonist SCH 23390 (Fig. 1*d*). These results match what was seen with the p-ERK 1/2, suggesting that the earlier signaling changes may be due to changes in β-arrestin 1 recruitment. This negative cross-talk and cross-antagonism at the level of β-arrestin 1 recruitment were not observed in cells treated with 30 μM cocaine (Fig. 1*d*), supporting the hypothesis that cocaine can alter the function of D$_1$R-H$_3$R heteromers. In the presence of cocaine, the imetit-induced β-arrestin 1 recruitment was very low (Fig. 1*d*) indicating the need for functional D$_1$R-H$_3$R heteromers for imetit to induce arrestin recruitment as well as ERK 1/2 signaling.
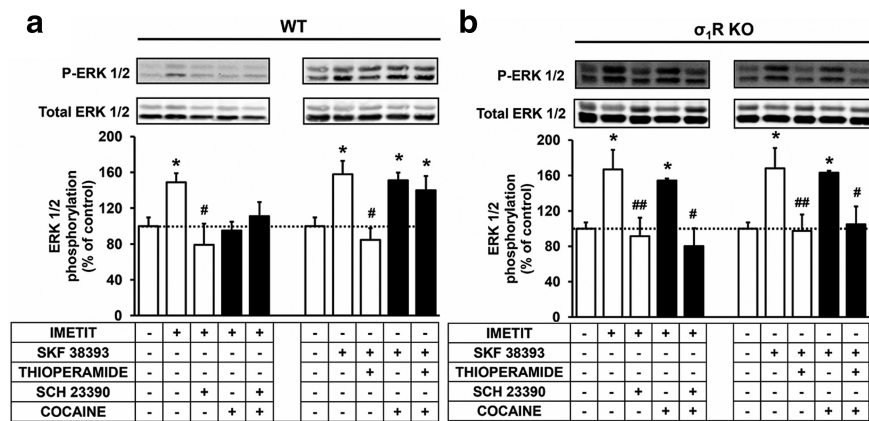
We and others have shown that one of the targets of cocaine is the chaperone-like protein, σ_1R, which can interact with and modulate D$_1$R (Hiranita et al., 2010, 2013; Navarro et al., 2010; Katz et al., 2011; Kourrich et al., 2012, 2013). From these studies it is tempting to hypothesize that σ_1R can mediate cocaine's effect by molecularly interacting with D$_1$R-H$_3$R heteromers. To test this, we first repeated the signaling in neuroblastoma cells using cells in which σ_1R was silenced by shRNA infection (Fig. 1*e*). In these cells, cocaine did not affect the cross-antagonism or the imetit-induced signaling (Fig. 1*f*), demonstrating that σ_1R are indeed mediating cocaine's effects on D$_1$R-H$_3$R signaling. These results show that σ_1R is required for cocaine's effects but does not demonstrate a molecular interac-

cross-antagonism was not seen in cells treated with 30 μM cocaine (Fig. 1*c*). Analogously, the H$_3$R agonist imetit-induced ERK 1/2 phosphorylation was reverted by both thioperamide and the D$_1$R antagonist SCH 23390. Again, the cross-antagonism was lost in the presence of cocaine and, in these conditions, the signaling induced by imetit was very low (Fig. 1*c*) in agreement with the requirement of D$_1$R-H$_3$R heteromers for imetit to signal via the ERK 1/2 pathway (Moreno et al., 2011a).
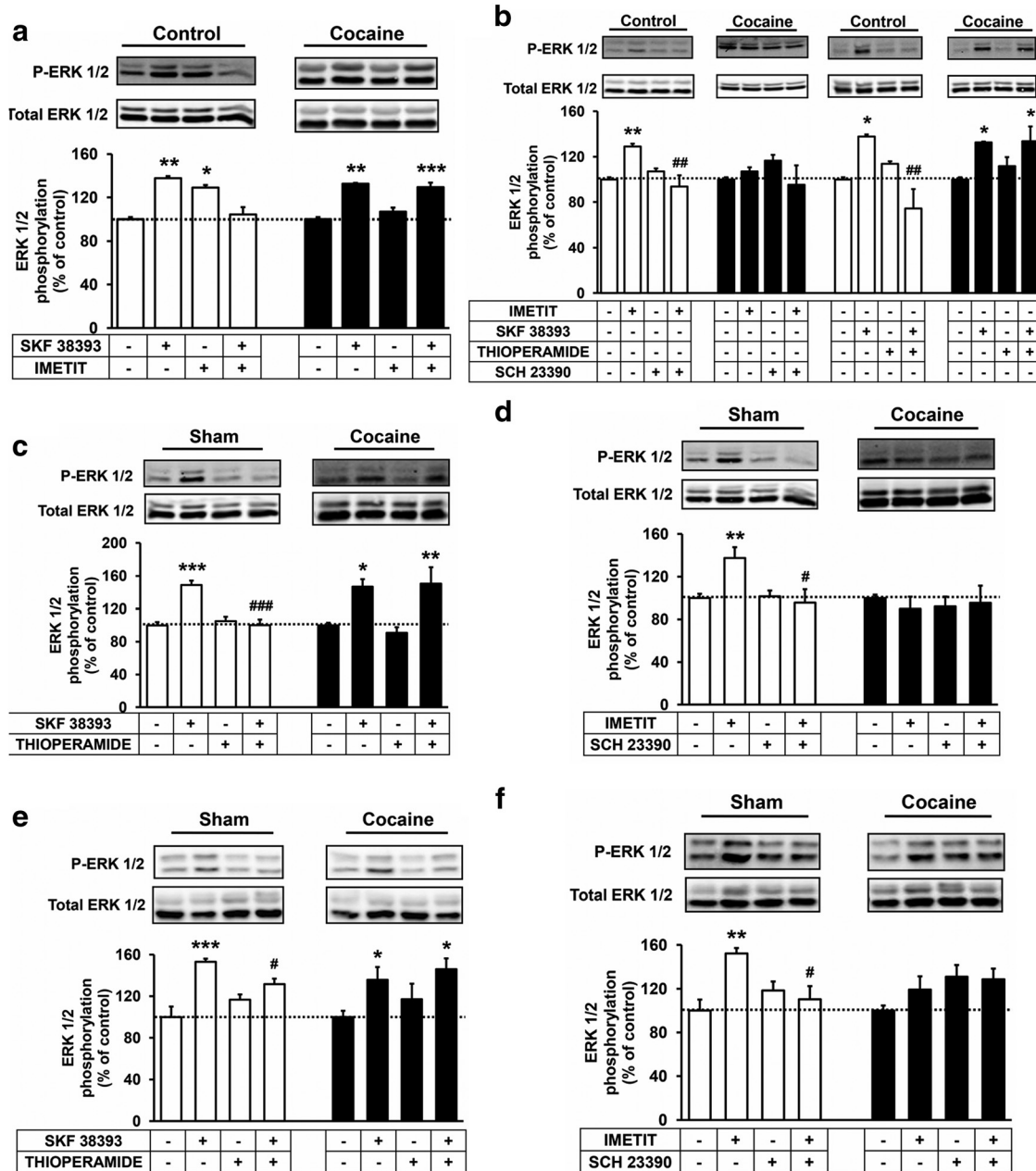
Figure 6. Cocaine inhibits the H$_3$R-mediated modulation of D$_1$R-promoted ERK 1/2 phosphorylation in rat striatum. Striatal slices from naive rats treated or not with 150 μM cocaine for 2 h (**a**, **b**) and striatal slices from sham rats or rats acutely treated (24 h) with cocaine (**c**, **d**), or striatal slices from sham rats or 9 weeks cocaine self-administered rats (**e**, **f**) were preincubated for 20 min with medium, the D$_1$R antagonist SCH 23390 (10 μM), or the H$_3$R antagonist thioperamide (10 μM) before the addition of the D$_1$R agonist SKF 38393 (1 μM) or the H$_3$R agonist imetit (1 μM) for an additional incubation period of 10 min and ERK 1/2 phosphorylation was determined by Western blot. Immunoreactive bands from 3 to 10 slices obtained from four animals were quantified for each condition. Values represent mean ± SEM percentage of phosphorylation relative to basal levels found in untreated slices. One-way ANOVA followed by Bonferroni *post hoc* tests showed a significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) effect over basal or of the antagonist plus agonist treatment over the agonist treatment ([#]$p < 0.05$, [##]$p < 0.01$, [###]$p < 0.001$).

tion between σ_1R and D$_1$R-H$_3$R heteromers. To test first if σ_1R can directly interact with H$_3$R we performed BRET experiments in HEK293T cells expressing a constant amount of H$_3$R-Rluc and increasing amounts of σ_1R-YFP. As shown in Figure 2*a*, we did not detect energy transfer in these cells nor in cells treated with 30 μM cocaine for 30 min, suggesting a lack of interaction between H$_3$R and σ_1R. Next we used SRET (Carriba et al., 2008) to study if H$_3$R and σ_1R can interact in the presence of D$_1$R. For this, Rluc was fused to H$_3$R to act as a BRET donor, GFP^2 was fused to D$_1$R to act as a BRET acceptor and as a FRET donor, and YFP was fused to σ_1R to act as a FRET acceptor (Fig. 2*b*). In HEK293T cells expressing a constant amount of H$_3$R-Rluc and D$_1$R-GFP^2 and

increasing amounts of σ_1R-YFP, a positive SRET saturation curve was obtained (Fig, 2*b*, red curve) with a SRET$_{max}$ of 114 ± 18 mSU and SRET$_{50}$ of 41 ± 18. These results indicate that σ_1Rs can complex with D$_1$R-H$_3$R heteromers through an interaction with D$_1$R in the heteromer. As cocaine can bind σ_1R at pharmacological relevant concentrations (Matsumoto et al., 2003; Hayashi and Su, 2005; Hiranita et al., 2010, 2013; Kourrich et al., 2012) we next wanted to examine whether cocaine is able to modulate the D$_1$R-H$_3$R heteromer at the molecular level via σ_1R. When the SRET experiments described above were performed in cells treated 30 min with 30 μM cocaine (Fig. 2*b*, black curve), a positive SRET saturation curve was obtained with a slight increase in

the maximum energy transfer (SRET$_{max}$ = 145 ± 19 mBU and SRET$_{50}$ = 48 ± 17), suggesting that cocaine binding changes the quaternary structure of the heteromer decreasing the distance or favoring the orientation between the donor and acceptor proteins. The cocaine-induced structural changes were also observed in BRET saturation curves for the D$_1$R-Rluc-H$_3$R-YFP pair in cells expressing a constant amount of D$_1$R-Rluc and increasing amounts of H$_3$R-YFP where there was an increase in energy transfer after cells were treated with 30 μM cocaine for 30 min (Fig. 2c, red curve; BRET$_{max}$ = 52 ± 2 mBU and BRET$_{50}$ = 14 ± 2 vs black curve, BRET$_{max}$ = 68 ± 6 mBU and BRET$_{50}$ = 16 ± 4). This effect was reverted in cells whose endogenous σ_1R expression was silenced using siRNA previously described (Navarro et al., 2010; Fig. 2c, green curve; BRET$_{max}$ = 49 ± 4 mBU and BRET$_{50}$ = 11 ± 3). Similar results were obtained in cells treated with 30 μM cocaine for 24 h (Fig. 2d). These results suggest that cocaine binding to σ_1R is not disrupting D$_1$R-H$_3$R heteromers, but is inducing structural changes in D$_1$R-H$_3$R heteromers. Thus, it is clear that in transfected cells cocaine, by binding to σ_1R, alters the D$_1$R-H$_3$R heteromer such that it can diminish H$_3$R receptor ability to modify D$_1$R signaling.

The σ_1R mediates cocaine's effects on D$_1$R-H$_3$R heteromer function in the striatum

Although the σ_1R-mediated ability of cocaine to modulate the structure and signaling of D$_1$R-H$_3$R heteromers in cells is suggestive of a novel cocaine mechanism, it is important to show that similar events can occur in tissue. First we tested whether σ_1R-D$_1$R-H$_3$R complexes are expressed in WT and σ_1R KO mice striatum by coimmunoprecipitation experiments and by proximity ligation assays (PLA) using specific antibodies we described previously (Moreno et al., 2011a; Navarro et al., 2013). By Western blot experiments we found the expression of both D$_1$R and H$_3$R in the striatum of WT and KO mice and the expression of σ_1R receptors in WT but not in the striatum of σ_1R KO mice (Fig. 3a). In coimmunoprecipitation experiments (Fig. 3b–d) the antibody against D$_1$R could indeed coprecipitate D$_1$R (Fig. 3b) and H$_3$R (Fig. 3c) in WT or KO mice striatal membranes, and this antibody also coimmunoprecipitates σ_1R from WT striatum but not from KO striatum (Fig. 3d). It is interesting to note that the D$_1$R-H$_3$R or D1R-σ_1R coimmunoprecipitation increased after treatment with 30 μM cocaine (Fig. 3c and d). This could be due to a cocaine-induced higher σ_1R expression at the membrane (Hayashi and Su, 2005; Navarro et al., 2010) where heterocomplex formation might be favored. The coimmunoprecipitation results are in line with the BRET experiments above and highly suggestive that σ_1R-D$_1$R-H$_3$R complexes are expressed in the striatum of WT animals and that D$_1$R-H$_3$R heteromer expression is maintained in the striatum of σ_1R KO mice. Interestingly, the coprecipitation of H$_3$R by anti-D$_1$R is more efficient in the presence of cocaine, suggesting a change in the strength of interaction, consistent with the increase in the SRET detected in Figure 2b. Furthermore we used the PLA to explore whether D$_1$R-H$_3$R heteromer expression was maintained in σ_1R KO mice and whether σ_1R-D$_1$R-H$_3$R complexes could be detected in WT mice. PLA requires that both receptors be close enough to allow the two different antibody-DNA probes to be able to ligate (<17 nm; Söderberg et al., 2008; Trifilieff et al., 2011). If the receptors are within sufficient proximity, a punctate fluorescent signal can be detected by confocal microscopy. Using striatal slices from both WT (Fig. 3e–g) and σ_1R KO (Fig. 3h–j), a red punctate fluorescent stain surrounding DAPI-positive nuclei was observed for the H$_3$R-D$_1$R (Fig. 3e), D$_1$R-σ_1R (Fig. 3f), or H$_3$R-σ_1R (Fig. 3g) pairs
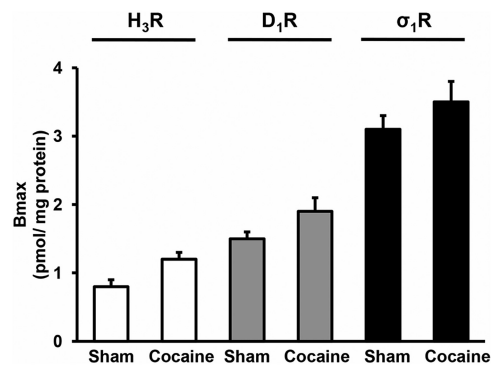

Figure 7. Receptor expression in the striatum of cocaine acutely treated rats compared with sham rats. The expression of H$_3$R (white bars), D$_1$R (gray bars), and σ_1R (black bars) in striatal membranes from a pool (n = 5) of sham rats or rats acutely treated (24 h) with cocaine (cocaine) was determined by competition experiments of [^{3}H]RAMH (3 nM) versus RAMH, [^{3}H]SCH 23390 (2 nM) versus SCH 23390, or [^{3}H] YM-09151-02 (2 nM) versus PRE-084, respectively. Competition curves were monophasic and binding data were fitted to Equations 4 or 5 (see Materials and Methods) to calculate the maximum binding.

in WT mouse striatum but only H$_3$R-D$_1$R heteromers were detected in σ_1R KO animals (Fig. 3h). These data confirm the coprecipitation experiments above. As a negative control we checked that the red spots were not seen in slices treated with only one primary antibody and both secondary antibody-DNA probes (Fig. 3 k–m). To determine the cocaine effect on the striatal D$_1$R-H$_3$R heteromerization we performed PLA experiments in the presence of cocaine. In these conditions, results from PLA experiments were very similar to the ones obtained in the absence of cocaine (results not shown), indicating that σ_1R, D$_1$R, and H$_3$R are indeed in a complex in mice striatum in the presence or absence of cocaine. Next, by using the coimmunoprecipitation and PLA experiments described above, we observed that σ_1R-D$_1$R-H$_3$R complexes are expressed in the rat striatum since the antibody against D$_1$R could indeed coprecipitate D$_1$R, H$_3$R, and σ_1R in the absence or the presence of cocaine and we detected red spots in the PLA experiments for the pairs H$_3$R-D$_1$R, D$_1$R-σ_1R, or H$_3$R-σ_1R (Fig. 4). These results suggest that σ_1R-D$_1$R-H$_3$R complexes are expressed in rat striatum and that cocaine, by binding to σ_1R, does not disrupt the heteromer per se.

To test the cocaine effect on H$_3$R-D$_1$R heteromer signaling in the striatum we determined the receptor agonists induced ERK 1/2 phosphorylation using striatal slices. To test if cocaine effect is mediated by σ_1R we performed the signaling experiments in WT and σ_1R KO mice. If cocaine is indeed acting via σ_1R to modify the D$_1$R-H$_3$R heteromer in the striatum, then we should see no effect of cocaine in the σ_1R KO mice. The striatal level of cocaine reached after pharmacologically significant doses is 15 μM (Pettit et al., 1990). To allow diffusion into the tissue in brain slices we used a 10-fold higher cocaine concentration, 150 μM. We determined the cross-antagonism in ERK 1/2 phosphorylation using striatal slices from WT and σ_1R KO mice in the absence or the presence of cocaine. In the absence of cocaine the ERK 1/2 phosphorylation induced by the D$_1$R agonist SKF 38393 was reverted by the H$_3$R antagonist thioperamide in striatal slices from WT (Fig. 5a) and from σ_1R KO mice (Fig. 5b). These data indicate that functional D$_1$R-H$_3$R heteromers are expressed in the striatum of both WT and σ_1R KO mice in agreement to the PLA results above. In addition, the presence of cocaine did not affect H$_3$R signaling and the cross-antagonism in striatal slices from σ_1R KO mice (Fig. 5b). In contrast, cocaine blocked the H$_3$R signaling and the D$_1$R-H$_3$R cross-antagonism in slices from

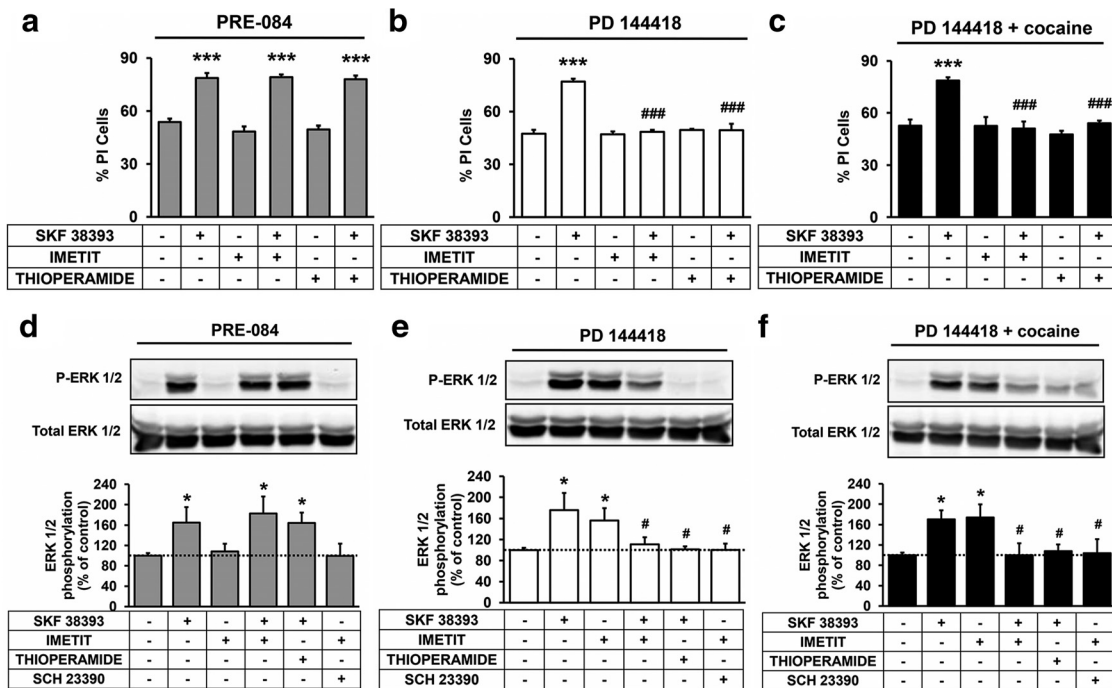**Figure 9.** Effect of σ₁R agonist and antagonists on the H₃R-mediated modulation of the D₁R-promoted cell death and signaling in the striatum. Rat striatal organotypic cultures (**a**–**c**) or striatal slices (**d**–**f**) were prepared as indicated (see Materials and Methods) and were treated for 1 h with medium (**a**, **d**) or PD 144418 (10 μM; **b**, **c**, **e**, **f**) before adding 1 μM PRE-084 (**a**, **d**), vehicle (**b**, **e**), or 150 μM cocaine (**c**, **f**). At 24 h of organotypic culture (**a**–**c**) or after 2 h of slice incubation (**d**–**f**) the D₁R antagonist SCH 23390 (50 μM, **a**–**c** or 10 μM, **d**–**f**) or the H₃R antagonist thioperamide (10 μM) were added 20 min before the addition of the D₁R agonist SKF 38393 (50 μM, **a**–**c** or 1 μM, **d**–**f**) or the H₃R agonist imetit (10 μM, **a**–**c** or 1 μM, **d**–**f**) alone or in combination. Striatal organotypic cultures were cultured for an additional 48 h and slices were incubated for an additional 10 min and were processed to determine cell death or ERK 1/2 phosphorylation, respectively, as indicated in Materials and Methods. **a**–**c**, Values represent mean ± SEM percentage of PI stained cells versus total DAPI-stained cells determined in four to six fields from two to three independent organotypic cultures. **d**–**f**, Values represent mean ± SEM phosphorylation relative to basal levels found in untreated slices, determined in 3–10 slices obtained from four animals. One-way ANOVA followed by Bonferroni *post hoc* tests showed a significant (*$p < 0.05$, ***$p < 0.001$) effect over basal (nontreated organotypic cultures or slices) or of the two ligands treatment over the agonist treatment ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$).

1). Since the effect was observed after a short treatment with cocaine, these results suggest that cocaine binding to σ₁R is blocking the allosteric interaction between H₃R and D₁R in the σ₁R-D₁R-H₃R complex.

Effect of cocaine in the H₃R-mediated modulation of D₁R-induced cell death in the striatum

The results above described indicate that H₃R can act as a "molecular brake" of D₁R function by forming heteromers in the rat striatum, blocking the ability of D₁R to signal and that cocaine can remove this brake and allow D₁R to signal unimpeded by H₃R. Ideally we would like to have a physiological readout of these changes in the D₁R-H₃R heteromer function. It has been shown that the dopamine increase due to drug intake is neurotoxic (Cunha-Oliveira et al., 2008) and it has been described that striatal cells are especially vulnerable to dopamine D₁R activation by a nonoxidative process (Paoletti et al., 2008). Here, using organotypic rat striatal cultures we tested the hypothesis that blocking D₁R signaling via H₃R could lead to a change in D₁R overactivation-induced cell death. We first tested the H₃R ligands effect on D₁R-induced cell death in organotypic rat striatal cultures. As indicated in Materials and Methods, we determined cell death by comparing DAPI and PI staining. The D₁R agonist SKF 38393, but not H₃R agonist imetit or the antagonist thioperamide, induced cell death, an effect that was reverted by the D₁R antagonist SCH 23390 (Fig. 8a). When SKF 38393 and imetit were added together to the organotypic cultures, imetit blocked the D₁R-mediated cell death (Fig. 8a) and the imetit effect was specific for D₁R-mediated cell death as it was not observed in

glutamate-induced cell death (Fig. 8b). Similar blocking of cell death was observed when SKF 38393 and thioperamide were added together (Fig. 8a). Thus, reducing D₁R signaling via H₃R can serve to reduce downstream physiological effects in our organotypic models, indicating that H₃R indeed serves as a "molecular brake" or control point for D₁R function. We next examined whether cocaine could remove this brake performing the same experiments in organotypic cultures treated with 150 μM cocaine. As shown in Figure 8c, cocaine was able to disrupt the imetit inhibition of the SKF 38393-promoted cell death without altering the effect of SKF 38393 alone and also blocked the cross-antagonism exerted by the H₃R antagonist thioperamide on SKF 38393-promoted cell death (Fig. 8c).

The results above suggest that cocaine's disruption of D₁R-H₃R signaling is an important enough disruption that can translate into pathological consequences. Thus, in cases where altering D₁R signaling might be desired, it could be advantageous to target D₁R-H₃R heteromers via σ₁R. To test this, we analyzed the effect of H₃R ligands on the D₁R agonist SKF 38393-induced cell death in organotypic cultures pretreated with σ₁R agonists and antagonists. The pretreatment with the agonist PRE-084 (1 μM) mimicked the effect of cocaine in the H₃R ligand modulation of D₁R-induced cell death (Fig. 9a) and the pretreatment with the antagonist PD 144418 (10 μM), which had no effect in this process (Fig. 9b), could block the 150 μM cocaine's effects on the D₁R-H₃R heteromer in our cell death model (Fig. 9c). We also analyzed the D₁R-H₃R cross-talk and cross-antagonism in signaling in rat striatal slices pretreated with 1 μM PRE-084 or 10 μM PD 144418 in the absence or in the presence of 150 μM cocaine.

As shown in Figure 9d, PRE-084 mimicked cocaine's effect by blocking the negative cross-talk between SKF 38393 and imetit in ERK 1/2 phosphorylation and the cross-antagonism between both receptors. When striatal slices where pretreated with the $\sigma 1$ receptor antagonist PD 144418 (10 μM) cocaine could no longer block H$_3$ receptor's control of D1 receptor signaling (Fig. 9f). PD 144418 had no effect in the H$_3$R ligand modulation of D$_1$R signaling (Fig. 9e) suggesting that it was indeed through blockage of cocaine binding. These data strongly suggest that H$_3$R control of D$_1$R signaling can be pharmacologically influenced with σ_1R compounds and that σ_1R antagonists may prove effective at reducing the effects of cocaine.

Discussion

From the data presented here four major conclusions can be made. First, σ_1R binds D$_1$R-H$_3$R heteromers in transfected cells as well as in mouse and rat striatum. Second, σ_1R agonists, such as cocaine, modify the structure and counteract the biochemical properties of the D$_1$R-H$_3$R heteromer, composing of heteromer signaling through G$_i$ protein, the ability of H$_3$R activation to signal through MAPK, and the ability of H$_3$R ligands to inhibit the effects of D$_1$R-mediated signaling, including cell death. Therefore, a third important general conclusion is that blockade of H$_3$R-mediated inhibition of D$_1$R function in the σ_1R-D$_1$R-H$_3$R complexes plays a key role in the effects of cocaine. Finally, σ_1R-D$_1$R-H$_3$R complexes might provide a new target for the treatment of cocaine abuse.

Cocaine binds with somewhat higher affinity to DAT than to σ_1R; therefore, the role of σ_1R in the addictive properties of cocaine has been a matter of debate. More accepted has been their role in the acute neurotoxic effects of cocaine (for review, see Robson et al., 2012). In fact, σ_1R antagonists provide a protective effect in cocaine-induced convulsions and lethality in rodents (Ritz and George, 1997; Robson et al., 2012). Nevertheless, an important amount of experimental data demonstrates the involvement of σ_1R in many pharmacologic, including rewarding, effects of cocaine (Maurice and Su, 2009; Robson et al., 2012). Repeated cocaine exposure increases the levels of σ_1R in the brain, including the striatum, by a process that depends on the cocaine binding σ_1R (Liu and Matsumoto, 2008), and does not occur in mice with genetic deletion of D$_1$R (Zhang et al., 2005). Upregulation of σ_1R might underlie the ability of cocaine to induce self-administration of selective σ_1R agonists, which has been invoked as a possible mechanism that contributes to the intractability of psychostimulant abuse (Hiranita et al., 2013). Indeed, we have demonstrated the importance of the σ_1R and D$_1$R receptor interaction on the initial events upon cocaine exposure (Navarro et al., 2010).

We previously described that in the striatum D$_1$R can form heteromers with H$_3$R and that H$_3$R agonist binding to the D$_1$R-H$_3$R heteromers are negatively modulating D$_1$R function (Ferrada et al., 2009; Moreno et al., 2011a). The modulation of D$_1$R
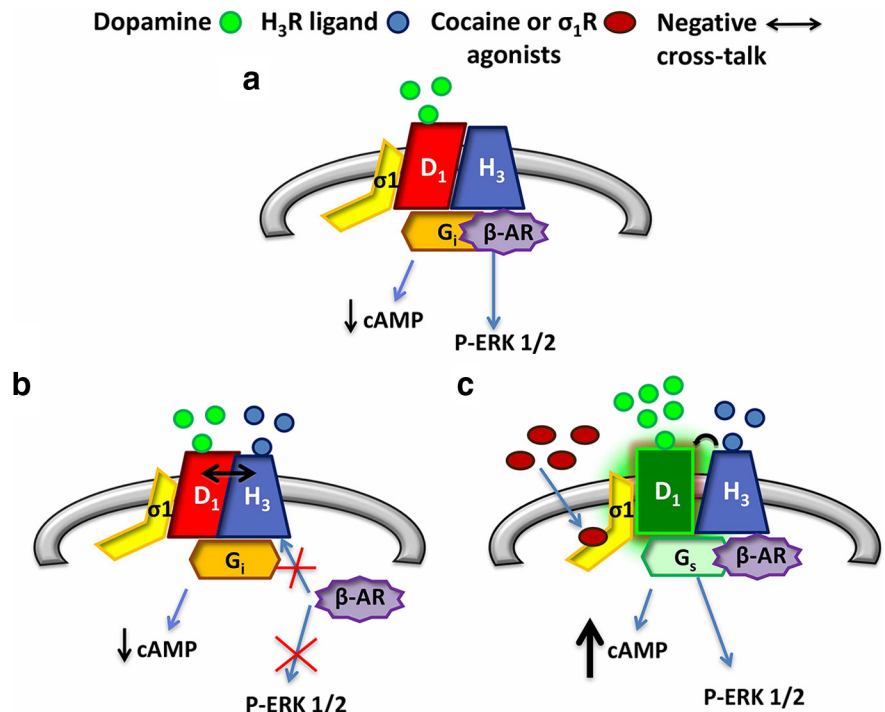

Figure 10. Scheme representing the D$_1$R signaling in the D$_1$R-H$_3$R heteromer in the absence or presence of cocaine. In GABAergic neurons (gray), in the absence of cocaine, H$_3$R (blue) and D$_1$R (red) form heteromers to which σ_1R (yellow) can bind. Activation of only the D$_1$R promoter (**a**) promotes a decrease of cAMP levels in accordance with G$_i$ (orange) coupling of both receptors in the heteromer. Moreover, D$_1$R activation induces β-arrestin (purple) recruitment and promotes ERK 1/2 phosphorylation. Upon dual stimulation of the heteromer with both H$_3$R and D$_1$R agonists in the absence of cocaine (**b**), a negative cross-talk (the ability of one receptor agonist to negatively modulate the partner receptor ligand binding or signaling) between receptors in the heteromer occurs, inhibiting β-arrestin recruitment and subsequently reducing ERK 1/2 phosphorylation from the heteromer. When cocaine (brown) binds to σ_1R (**c**) this induces structural changes in the heteromer disrupting the negative cross-talk. Thus, the H$_3$R-mediated brake on D$_1$R is lost, allowing D$_1$R (green) to couple to G$_s$ (pale green) and increasing cAMP. Importantly, β-arrestin recruitment and ERK 1/2 phosphorylation are also detected in the presence of cocaine.

signaling by H$_3$R is thought to serve as a control point that could set the tone of D$_1$R signaling from a given neuron (Moreno et al., 2011a; Ellenbroek, 2013; Panula and Nuutinen, 2013). However, previous studies using the H$_3$R ligand imetit have shown that it was not effective at preventing drug seeking in the presence of cocaine, but does prevent drug seeking in the absence of cocaine (Ortiz De Pablo and Self, 2011). Our work here clearly explains the molecular mechanism behind these results and highlights the pleiotropic effects of σ_1R. As stated in Figure 10, H$_3$R and D$_1$R form heteromers to which σ_1R can bind. Under normal conditions (in the absence of cocaine), H$_3$R can serve to control the levels of D$_1$R signaling altering its coupling to G-proteins. Activating only the D$_1$R protomer in the heteromer promotes a decrease of cAMP levels in accordance with G$_i$ coupling to the heteromer. In addition, D$_1$R activation induces β-arrestin recruitment and promotes ERK 1/2 phosphorylation (Fig. 10a). However, upon dual stimulation of the heteromer with both H$_3$R and D$_1$R agonists in the absence of cocaine, a negative cross-talk between receptors in the heteromer occurs, inhibiting β-arrestin recruitment and subsequently reducing ERK 1/2 phosphorylation from the heteromer (Fig. 10b). When cocaine (Fig. 10c) binds to σ_1R in the heteromeric complex, it induces structural changes in the heteromer, and disrupts the negative cross-talk. Thus, in the presence of cocaine the H$_3$R-mediated brake on D$_1$R is lost. Importantly, cocaine also blocks the dopamine transporter, DAT, in presynaptic terminals. This leads to an increase in extracellular levels of dopamine. This dopamine then can bind

and activate an uninhibited D$_1$R allowing D$_1$R to increase cAMP by coupling to G$_s$ protein and increasing p-ERK 1/2, presumably via recruitment of β-arrestin. This uninhibited D$_1$R is normally balanced by inputs from D$_2$R-containing neurons. However, we and others have shown that this balance becomes undone in the presence of cocaine (Navarro et al., 2013; Park et al., 2013).

The present study supports the use of σ_1R antagonists as a treatment for cocaine abuse, particularly in the frame of σ_1R modulation of D$_1$R-H$_3$R heteromers. In fact, we showed that blockade of σ_1R restores the protective H$_3$R-mediated brake on D$_1$R signaling involved in cocaine-induced cell death. Although at the experimental level D$_1$R seems to be clearly involved in the pharmacological effects of cocaine (Xu et al., 1994; Weiss et al., 2001; Wolf et al., 2003; Bertran-Gonzalez et al., 2008), conflicting results of D$_1$R antagonists have been observed at the clinical level (Nann-Vernotica et al., 2001), which might be related to the inability to reach therapeutic doses without secondary effects. Antagonists of σ_1R receptors can block cocaine's ability to modulate D$_1$-H$_3$ receptor heteromers, providing a means to decrease the overdose-related side effects of orthosteric D$_1$ receptor antagonists. Our results indicate that a potentially good therapeutic approach for the pharmacologic effects of cocaine could include the association of σ_1R antagonists with H$_3$ receptor agonists.

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