



# Cocaine Effects on Dopaminergic Transmission Depend on a Balance between Sigma-1 and Sigma-2 Receptor Expression

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Sigma  $\sigma_1$  and  $\sigma_2$  receptors are targets of cocaine. Despite sharing a similar name, the two receptors are structurally unrelated and their physiological role is unknown. Cocaine increases the level of dopamine, a key neurotransmitter in CNS motor control and reward areas. While the drug also affects dopaminergic signaling by allosteric modulations exerted by  $\sigma_1$ R interacting with dopamine  $D_1$  and  $D_2$  receptors, the potential regulation of dopaminergic transmission by  $\sigma_2$ R is also unknown. We here demonstrate that  $\sigma_2$ R may form heteroreceptor complexes with  $D_1$  but not with  $D_2$  receptors. Remarkably  $\sigma_1$ ,  $\sigma_2$ , and  $D_1$  receptors may form heterotrimers with particular signaling properties. Determination of cAMP levels, MAP kinase activation and label-free assays demonstrate allosteric interactions within the trimer. Importantly, the presence of  $\sigma_2$ R induces bias in signal transduction as  $\sigma_2$ R ligands increase cAMP signaling whereas reduce MAP kinase activation. These effects, which are opposite to those exerted via  $\sigma_1$ R, suggest that the  $D_1$  receptor-mediated signaling depends on the degree of trimer formation and the differential balance of sigma receptor and heteroreceptor expression in acute versus chronic cocaine consumption. Although the physiological role is unknown, the heteroreceptor complex formed by  $\sigma_1$ ,  $\sigma_2$ , and  $D_1$  receptors arise as relevant to convey the cocaine actions on motor control and reward circuits and as a key factor in acquisition of the addictive habit.

**Keywords:** acute, addiction, cAMP, chronic, dopamine  $D_1$  and  $D_2$  receptors, ERK1/2 phosphorylation, label-free, signaling

## INTRODUCTION

In advanced societies cocaine addiction is an important health and socio-economic problem. Cocaine use begins recreationally and the seeking behavior is based on a feeling of general well-being. Drug addiction is the result of plastic changes in areas of the brain that have dopamine as the main neurotransmitter, particularly in the *ventral tegmental area* (VTA) (see Lüscher, 2013 and references therein). The main consequence of cocaine consumption in the central nervous system (CNS) is an increase in interneuronal dopamine levels, which is not limited to VTA but extends

to other structures, such as the basal ganglia (Wise, 1984; Bradberry, 2008). It was thought that the inhibition of dopamine transporters was at the root of all the effects caused by this drug of abuse. However, there is strong evidence showing that cocaine exerts effects by a direct interaction with sigma receptors. Two different sigma receptors have been identified that are functionally and structurally unrelated. Although endogenous ligands are not known and the physiological function of sigma receptors is unclear, these receptors share the ability to bind cocaine. On the one hand, sigma-1 receptor ( $\sigma_1R$ ) is a chaperone that spans once the membrane bilayer and whose recently reported structure consists of a homotrimer (Schmidt et al., 2016). On the other hand, sigma-2 receptor ( $\sigma_2R$ ) was identified as a member of the family of membrane-associated progesterone receptors; apart from  $\sigma_2R$  (PGRMC1), three other human members are identified: PGRMC2, neuferricin, and neudesin. They are haem proteins displaying a cytochrome b<sub>5</sub>-fold domain. While  $\sigma_2R$  dimerization affects proliferation and chemoresistance in tumor/metastasis *in vitro* models and xenograft-based tumor/metastasis models, the mode of action in the periphery and the CNS are virtually unknown (Kabe et al., 2016; reviewed in Cahill, 2017). The interaction of  $\sigma_1R$  with dopamine receptors and the relevant role that  $\sigma_1R$  exerts on the modulation of dopaminergic signaling by cocaine has been reported. In contrast, no study has been undertaken to know whether the binding of cocaine to  $\sigma_2R$  results in dopaminergic regulation.

The role of  $\sigma_1R$  as relevant target of cocaine was suspected due to the moderate affinity of drug binding to the receptor (Matsumoto et al., 2003; Hayashi and Su, 2005). Therefore, it seems that the “physiologically” relevant concentrations of cocaine can both inhibit the uptake of dopamine and activate  $\sigma_1R$ .  $\sigma_1R$ -cocaine interaction intervenes in the triggering of locomotor and convulsive actions of the drug (Menkel et al., 1991; Matsumoto et al., 2001a,b, 2002; Barr et al., 2015). In addition, synthetic drugs that act as  $\sigma_1R$  agonists and antagonists, respectively, potentiate (Matsumoto et al., 2002, 2003) and reduce (Matsumoto et al., 2004) cocaine actions. More recent studies have identified in both heterologous expression systems and natural sources an interaction between  $\sigma_1R$  and dopamine receptors (Navarro et al., 2010; Moreno et al., 2014; Borroto-Escuela et al., 2017). Accordingly, it has been suggested that dual antagonism of  $\sigma_1R$  and inhibition of the dopamine DAT transporter can effectively block cocaine self-administration (Katz et al., 2016). Matsumoto et al. (2007) reported that treatment with synthetic drugs that act on  $\sigma_2R$  attenuates cocaine-derived behavior in mice. Although the selectivity of the compounds was poor, 1 year later, Mésangeau et al. (2008) designed an approach for converting selective  $\sigma_1R$  ligands into  $\sigma_2R$  selective ligands that, importantly, showed anti-cocaine activity. Furthermore, it has been observed that treatment with  $\sigma_2R$  antagonists counteract locomotor stimulation induced by cocaine in mice (Lever et al., 2014; Guo and Zhen, 2015).

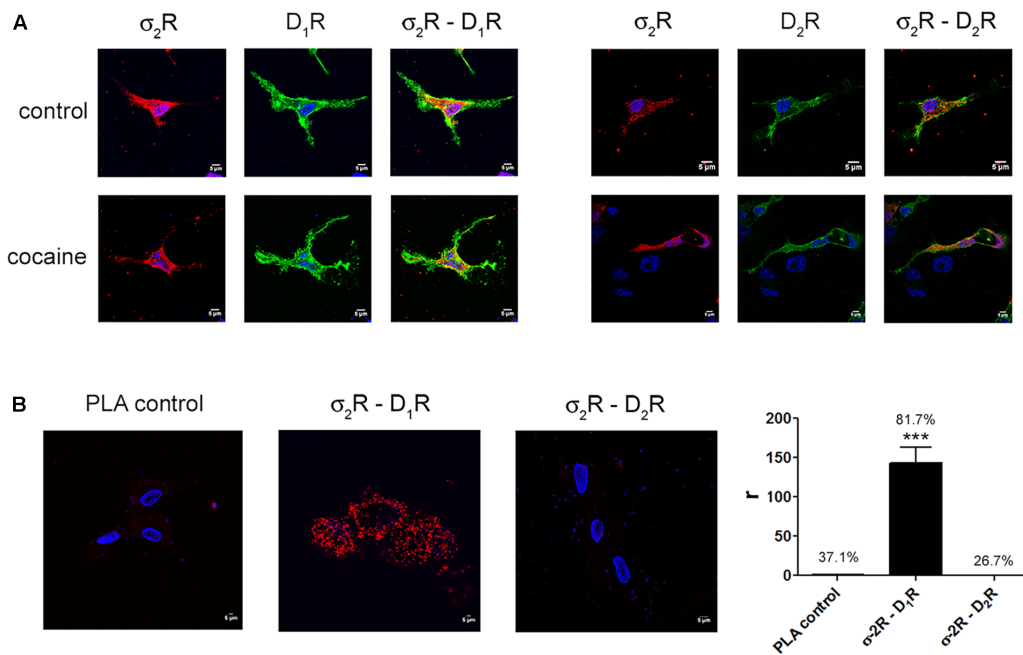
An important physiological consequence of cocaine ingestion is an increase in motor activity, which is controlled by basal-ganglia brain circuits. Motor control is exerted by the direct and indirect pathways of the basal ganglia and associated nuclei.

Of the five types of dopamine receptors, the D<sub>1</sub> (D<sub>1</sub>R) is enriched in the direct pathway, while the D<sub>2</sub> (D<sub>2</sub>R) is enriched in the indirect route. The balance of the dopaminergic input in the two circuits results in fine-tuning motor control. The locomotor hyperactivity resulting from cocaine use probably reflects a lack of balance in these two routes. The objective of this work was to investigate how the binding of cocaine to  $\sigma_2R$  affects dopaminergic signaling mediated by D<sub>1</sub>R and/or D<sub>2</sub>R. We first investigated whether  $\sigma_2R$  interacts with D<sub>1</sub>R or with D<sub>2</sub>R and, subsequently, we observed how cocaine could affect in a  $\sigma_1R$ -independent but  $\sigma_2R$ -dependent fashion the signal transduction triggered by agonist activation of D<sub>1</sub>R but not of D<sub>2</sub>R.

## RESULTS

### $\sigma_2R$ May Form Complexes with Dopamine D<sub>1</sub> But Not with Dopamine D<sub>2</sub> Receptors

Two different sigma receptors have been described, the non-opioid receptor,  $\sigma_1R$ , and the PGRMC-1 protein, also known as  $\sigma_2$ . Despite the endogenous ligands are not known, the two sigma receptors may bind cocaine. While recent studies have demonstrated that  $\sigma_1R$  is involved in cocaine modulation of dopamine receptor function, a similar study on  $\sigma_2R$ -mediated modulation of dopaminergic signaling is lacking. We first evaluated in a heterologous expression system whether  $\sigma_2R$  may colocalize with dopamine receptors at the plasma membrane. Immunocytochemistry assays were undertaken in HEK-293T cells expressing  $\sigma_2R$  fused to Rluc and either dopamine D<sub>1</sub>R fused to YFP or dopamine D<sub>2</sub>R fused to YFP. The  $\sigma_2R$  expression was identified by a specific antibody against Rluc protein and a secondary Cy3 antibody, while dopamine receptor-YFP expression was identified by its own fluorescence. D<sub>1</sub>R (green) was detectable at the plasma membrane level while  $\sigma_2R$  (red) was expressed both in intracellular structures and at the plasma membrane, where it colocalized (yellow) with D<sub>1</sub>R (Figure 1A, left images). When a similar experiment was developed with D<sub>2</sub>R, similar results were obtained indicating that D<sub>2</sub>R and  $\sigma_2R$  colocalize at the cell surface (Figure 1A, right images). When the immunocytochemical assays were performed in cells pretreated with 30  $\mu$ M cocaine for 30 min, the level of colocalization between  $\sigma_2R$  and D<sub>1</sub>R or D<sub>2</sub>R was similar, indicating that cocaine pretreatment did affect neither cell surface expression of  $\sigma_2R$ , D<sub>1</sub>R or D<sub>2</sub>R nor receptor colocalization. Next, we determined whether  $\sigma_2R$  may form heteromer complexes with dopamine D<sub>1</sub> or D<sub>2</sub> receptors. For this purpose, we took advantage of energy transfer assays and *in situ* proximity ligation assay (PLA), which allows the identification of close proximity between two proteins (<17 nm) (Borroto-Escuela et al., 2011; Trifilieff et al., 2011). For PLA, HEK-293T cells expressing  $\sigma_2R$  and either D<sub>1</sub>R or D<sub>2</sub>R were treated with specific primary antibodies against  $\sigma_2R$  and against each of the dopamine receptors. Interestingly, the red punctuated signal around Hoechst-stained nuclei was much higher for D<sub>1</sub>R and  $\sigma_2R$  than for D<sub>2</sub>R and  $\sigma_2R$  (82 versus 27% of labeled cells) (Figure 1B). Finally,



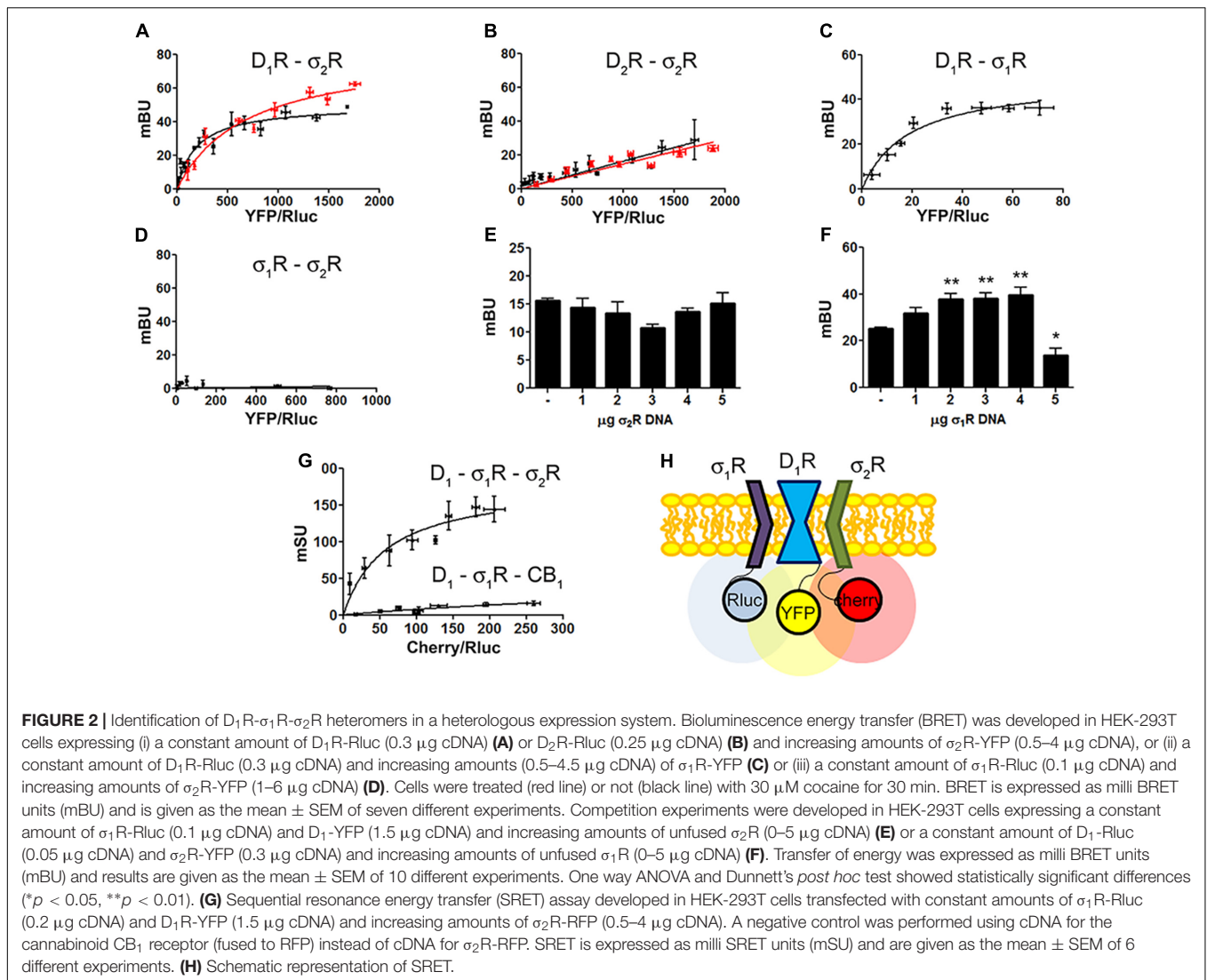
**FIGURE 1 |** Expression of  $\sigma_2R$ -containing heteromer complexes in a heterologous expression system. To determine colocalization between  $\sigma_2R$  and dopamine  $D_1$  or  $D_2$  receptors, immunocytochemistry assays were performed in HEK-293T pretreated or not with 30  $\mu M$  cocaine for 30 min. HEK-293T cells expressing  $\sigma_2R$ -Rluc (1  $\mu g$  cDNA),  $D_1R$ -YFP (1  $\mu g$  cDNA),  $D_2R$ -YFP (1  $\mu g$  cDNA),  $\sigma_2R$ -Rluc (1  $\mu g$  cDNA) and  $D_1R$ -YFP (1  $\mu g$  cDNA) or  $\sigma_2R$ -Rluc (1  $\mu g$  cDNA) and  $D_2R$ -YFP (1  $\mu g$  cDNA) were used. Dopamine receptors were detected by YFP fluorescence (green) and  $\sigma_2R$  was detected by a specific antibody against Rluc (1/100, Millipore, Temecula, CA, United States) followed by a Cy3-conjugated secondary antibody (1/200, Jackson ImmunoResearch Laboratories, West Grove, PA, United States) (red). Colocalization is shown in yellow (A). Scale bar 5  $\mu m$ . *In situ* proximity ligation assay (PLA) was developed in HEK-293T cells expressing  $D_1R$  (1  $\mu g$  cDNA) or  $D_2R$  (1  $\mu g$  cDNA) and  $\sigma_2R$  (1  $\mu g$  cDNA) by the use of specific primary antibodies (1/100 dilution) against  $D_1R$ ,  $D_2R$  and/or  $\sigma_2R$ . Nuclei were stained with Hoechst (1/100). Confocal microscopy images (four superimposed sections) were obtained showing  $D_1R$ - $\sigma_2R$  or  $D_2R$ - $\sigma_2R$  complexes as red spots (B). Scale bar 5  $\mu m$ . Quantification of the PLA provides in the Y-axis the ratio *r* (number of red spots/cell containing spots) and, above each bar, the percentage of positive cells versus the total number of cells (blue nucleus). Data are the mean  $\pm$  SEM of four different fields in five independent preparations. One way ANOVA and Dunnett's *post hoc* test showed statistically significant differences (\*\*\*)  $p < 0.001$ .

we developed bioluminescence energy transfer assays in HEK-293T cells transfected with cDNAs for  $D_1R$ -Rluc or  $D_2R$ -Rluc and increasing amounts of cDNA for  $\sigma_2R$ -YFP. Interestingly, a saturable BRET curve was obtained (BRET<sub>max</sub> 50  $\pm$  3, BRET<sub>50</sub> 190  $\pm$  40) (Figure 2A) indicating a specific interaction between  $D_1R$ - $\sigma_2R$ ; in contrast, a linear signal was obtained between  $D_2R$ - $\sigma_2R$  (Figure 2B) suggesting a lack of interaction between them. When the same experiments were undertaken in cells treated with cocaine, similar results were obtained for the  $D_1R$ -Rluc/ $\sigma_2R$ -YFP donor/acceptor pair (BRET<sub>max</sub> 82  $\pm$  10, BRET<sub>50</sub> 680  $\pm$  200), indicating that cocaine did not significantly affect the interaction (Figure 2B).

### Dopamine $D_1R$ , $\sigma_1R$ , and $\sigma_2R$ May Form Heterotrimeric Complexes

Dopamine  $D_1$  and  $\sigma_1$  receptors may form heteromeric complexes in HEK-293T cells (Navarro et al., 2010). To confirm whether in our experimental conditions  $D_1R$ -Rluc may act as a donor of  $\sigma_1R$ -YFP, BRET experiments undertaken in cotransfected HEK-293T cells provided a saturable curve thus indicating the interaction between  $\sigma_1R$  and  $D_1R$  (Figure 2C). We then hypothesized that  $\sigma_1R$  and  $\sigma_2R$  could be interacting together. Accordingly, BRET assays were performed in HEK-293T cells

expressing a constant amount of  $\sigma_1R$ -Rluc and increasing amounts of  $\sigma_2R$ -YFP. The unspecific linear signal obtained (Figure 2D) suggested that no interaction was occurring between the two sigma receptors. We then performed assays to investigate whether  $\sigma_1R$  and  $\sigma_2R$  competed for the binding to  $D_1R$ . BRET experiments were then developed in HEK-293T cells expressing a constant amount of  $\sigma_1R$ -Rluc and  $D_1R$ -YFP and increasing amounts of non-fused  $\sigma_2R$ . The results indicated that  $\sigma_2R$  was not able to compete with  $\sigma_1R$  for heteromer formation since the energy transfer between donor and acceptor was not altered (Figure 2E). When a similar experiment was performed expressing a constant amount of  $D_1R$ -Rluc and of  $\sigma_2R$ -YFP and increasing amounts of non-fused  $\sigma_1R$ , the results indicated that low expression levels of  $\sigma_1R$  increased BRET signals; however, higher expression levels  $\sigma_1R$  were able to displace  $\sigma_2R$  out of the heteromer, as reflected by a significant decrease in BRET signal (Figure 2F). This result could reflect the formation of  $D_1R$ - $\sigma_1R$ - $\sigma_2R$  heterotrimer complexes, where the interaction of  $\sigma_1R$  to the  $\sigma_2R$ - $D_1R$  complex could create a structural change in turn leading to increasing the energy transfer between Rluc and YFP. To confirm this possibility, sequential resonance energy transfer (SRET) assays, which permits detection of trimers (Carriba et al., 2008), were developed in HEK-293T

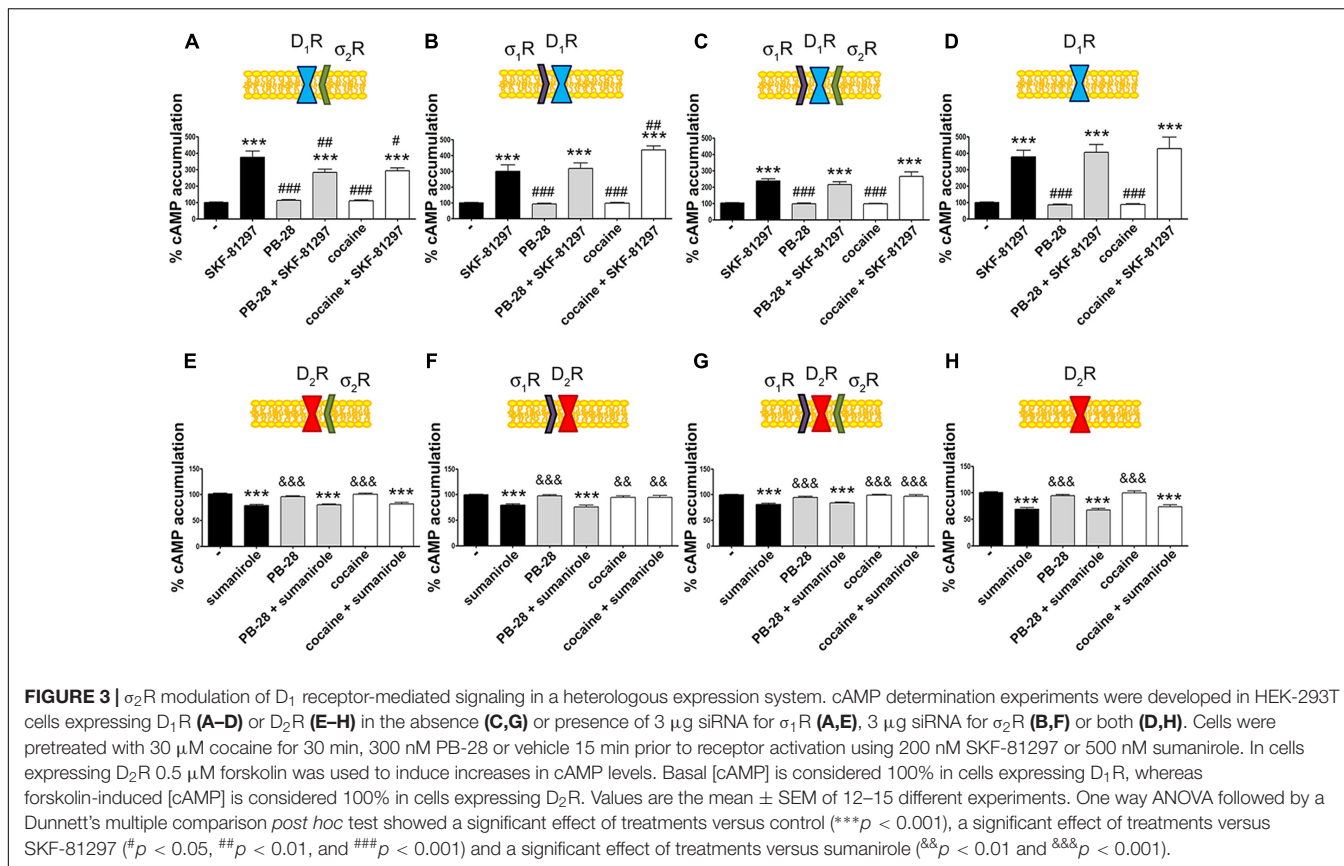


cells expressing a constant amount of  $\sigma_1R$ -Rluc and of  $D_1R$ -YFP and increasing amounts of  $\sigma_2R$ -Cherry. The saturable SRET curve indicates that formation of  $\sigma_1R$ - $D_1R$ - $\sigma_2R$  heteromer complexes was occurring (**Figures 2G,H**). The negative control was performed by substituting  $\sigma_2R$ -RFP by the cannabinoid  $CB_1$  receptor fused to RFP thus confirming the specificity of the triple  $\sigma_1R$ - $D_1R$ - $\sigma_2R$  interaction (**Figure 2G**).

## $\sigma_2R$ Activation Blocks Dopamine $D_1R$ Signaling

Our next aim was to characterize the functionality of the  $\sigma_1R$ - $D_1R$ - $\sigma_2R$  heterotrimer structure in HEK-293T cells treated with cocaine. It should be noted that  $\sigma_1R$  (Navarro et al., 2010) and  $\sigma_2R$  (Johannessen et al., 2011) are endogenously expressed in HEK-293T cells; consequently, we used a siRNA approach to silence  $\sigma_1R$  or  $\sigma_2R$  expression thus impeding heterotrimer formation. When HEK-293T cells were transfected with  $D_1R$  and siRNA for  $\sigma_1R$ , SKF-81297-induced a significant increase in cAMP levels, that was inhibited by pretreatment with cocaine or with the  $\sigma_2R$

agonist, PB-28, indicating that cocaine decreases  $D_1R$ -mediated cAMP signaling function through its binding to  $\sigma_2R$  (**Figure 3A**). When HEK-293T cells were transfected with  $D_1R$  and siRNA for  $\sigma_2R$ , the results indicated that cocaine pretreatment potentiated agonist-induced cAMP levels, which was evidence of cocaine action upon binding to the  $\sigma_1R$  (**Figure 3B**). The next set of results is consistent with a reciprocal modulation of signaling mediated by cocaine binding to  $\sigma_2R$  and  $\sigma_1R$ ; while cocaine via  $\sigma_1R$  positively modulates cAMP levels, it inhibits cAMP signaling via  $\sigma_2R$ . Accordingly, no effect of cocaine was observed in HEK-293T expressing  $D_1R$  and the two endogenous sigma receptors (**Figure 3C**). The lack of modulation exerted by cocaine upon simultaneous binding to both  $\sigma_1R$  and  $\sigma_2R$  likely reflects a balance which would, in a physiological set-up, depend on the relative expression of the two sigma receptors. In fact, when HEK-293T cells were transfected with  $D_1R$  and both siRNA for  $\sigma_1R$  and  $\sigma_2R$ , cocaine or the specific  $\sigma_2R$  agonist, PB-28, had no effect, indicating that cocaine modulation over  $D_1R$  depends on  $\sigma_1R$  and  $\sigma_2R$  expression (**Figure 3D**).

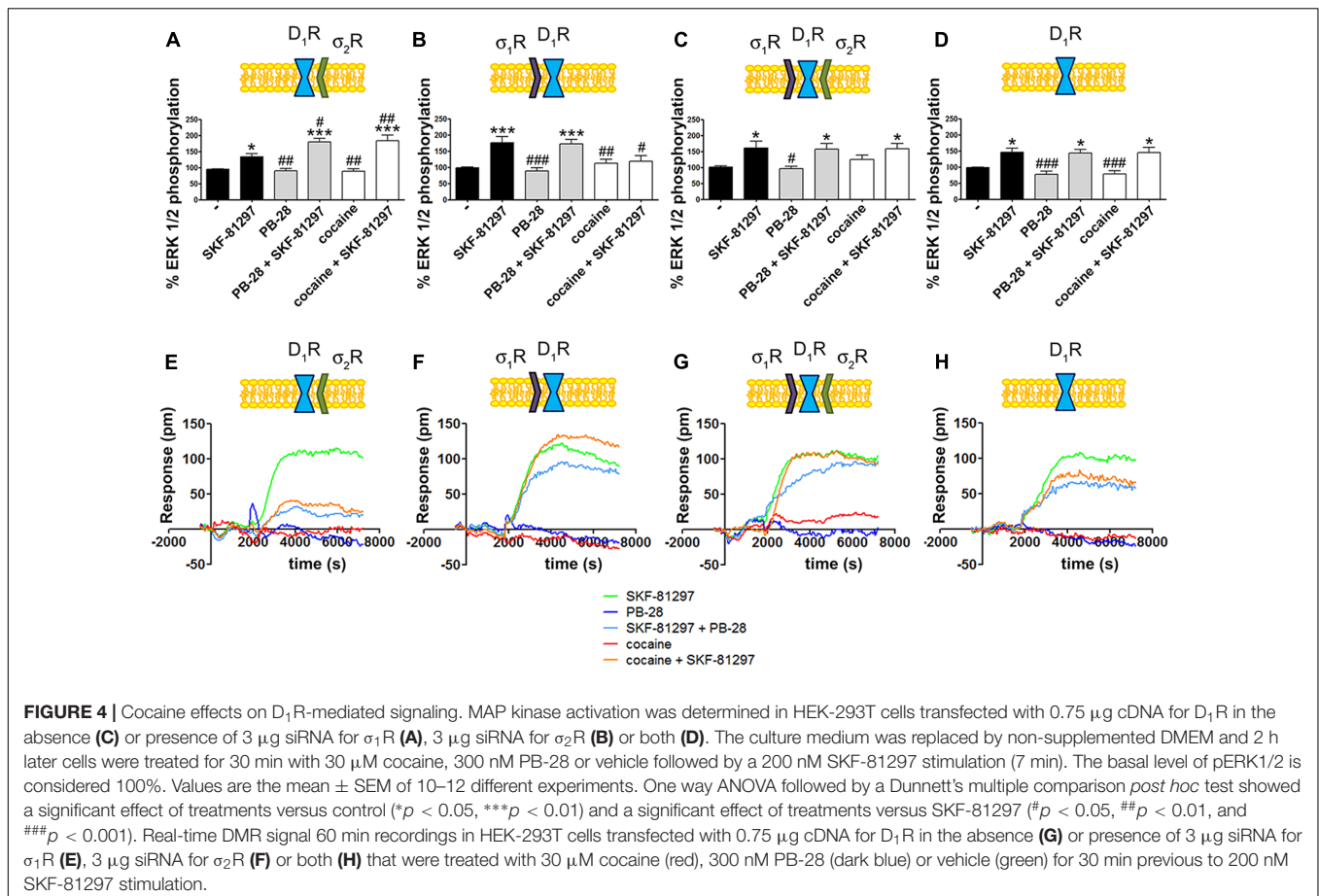


We next investigated whether cocaine binding to  $\sigma_2$ R receptors could still modulate D<sub>2</sub>R-mediated signaling. HEK-293T cells transfected with cDNAs for D<sub>2</sub>R and siRNA for  $\sigma_1$ R, responded to the selective-D<sub>2</sub>R agonist, sumanrirole. In these cells the G<sub>i</sub>-mediated decrease of forskolin-induced cAMP accumulation due to G<sub>i</sub> coupling was not affected by cocaine pretreatment (Figure 3E). These results agree with the lack of interaction between  $\sigma_2$ R and dopamine D<sub>2</sub>R (see Figure 2B). As a control, we confirmed that when the  $\sigma_1$ R-cocaine modulation over D<sub>2</sub>R was assayed, i.e., silencing  $\sigma_2$ R expression, cocaine was able to block the sumanrirole-induced effect (Figure 3F). These results agree with those in Navarro et al. (2013) in the sense that they reflect the consequence of a physical interaction between  $\sigma_1$ R and D<sub>2</sub>R receptors. In agreement with this hypothesis, HEK-293T cells expressing D<sub>2</sub>R and endogenous sigma receptors behaved as cells in which the  $\sigma_2$ R was silenced (Figure 3G). As a further control, HEK-293T cells treated with siRNAs to silence both sigma receptors showed no modulation by cocaine over D<sub>2</sub>R-mediated signaling (Figure 3H), thus reinforcing the idea that cocaine effect over D<sub>2</sub>R depends on  $\sigma_1$ R expression.

### $\sigma_2$ R Activation Potentiates Dopamine D<sub>1</sub>R MAP Kinase Phosphorylation

To further understand the cocaine effect over D<sub>1</sub>R function, MAP kinase signaling was evaluated in HEK-293T cells transfected with cDNAs for D<sub>1</sub>R and siRNA for either  $\sigma_1$ R or  $\sigma_2$ R. In

cells expressing D<sub>1</sub>R with silenced  $\sigma_1$ R, i.e., expressing D<sub>1</sub>R and  $\sigma_2$ R (Figure 4A), cocaine pretreatment increased agonist (SKF-81297)-induced ERK1/2 phosphorylation, while in cells with silenced  $\sigma_2$ R, i.e., expressing D<sub>1</sub>R and  $\sigma_1$ R (Figure 4B), cocaine decreased agonist-induced ERK1/2 phosphorylation. These results are evidence of potentiation by cocaine- $\sigma_2$ R of MAP kinase signaling, and potentiation by cocaine- $\sigma_1$ R of G<sub>s</sub>-protein dependent signaling. In cells expressing D<sub>1</sub>R and the two sigma receptors, no effect of cocaine pretreatment on pERK1/2 levels was observed, in agreement with the above-described balance resulting from reciprocal sigma-receptor-mediated cocaine effects (Figure 4C). As a further control, cocaine did not alter the SKF-81297-induced ERK1/2 phosphorylation in HEK-293T cells expressing D<sub>1</sub>R and with silenced sigma receptors (Figure 4D). A similar experimental design was used to undertake dynamic mass redistribution (DMR) assays. DMR is a label-free technique useful to investigate the activation of G-protein coupled receptors (Grundmann and Kostenis, 2015; Medrano et al., 2017). On the one hand, in cells expressing D<sub>1</sub>R and  $\sigma_2$ R, cocaine blocked SKF-81297-induced increase in the DMR signal in a similar way as the selective  $\sigma_2$ R ligand, PB-28, did (Figure 4E). On the other hand, the SKF-81297 effect was potentiated by cocaine pretreatment in cells expressing D<sub>1</sub>R and  $\sigma_1$ R (Figure 4F). Once more, cocaine modulation on D<sub>1</sub>R-agonist-induced effects was not found in cells expressing D<sub>1</sub>R and both sigma receptors (Figure 4G). As DMR in cells expressing D<sub>1</sub>R mainly reflects G<sub>s</sub>-coupling (Kebig et al., 2009;



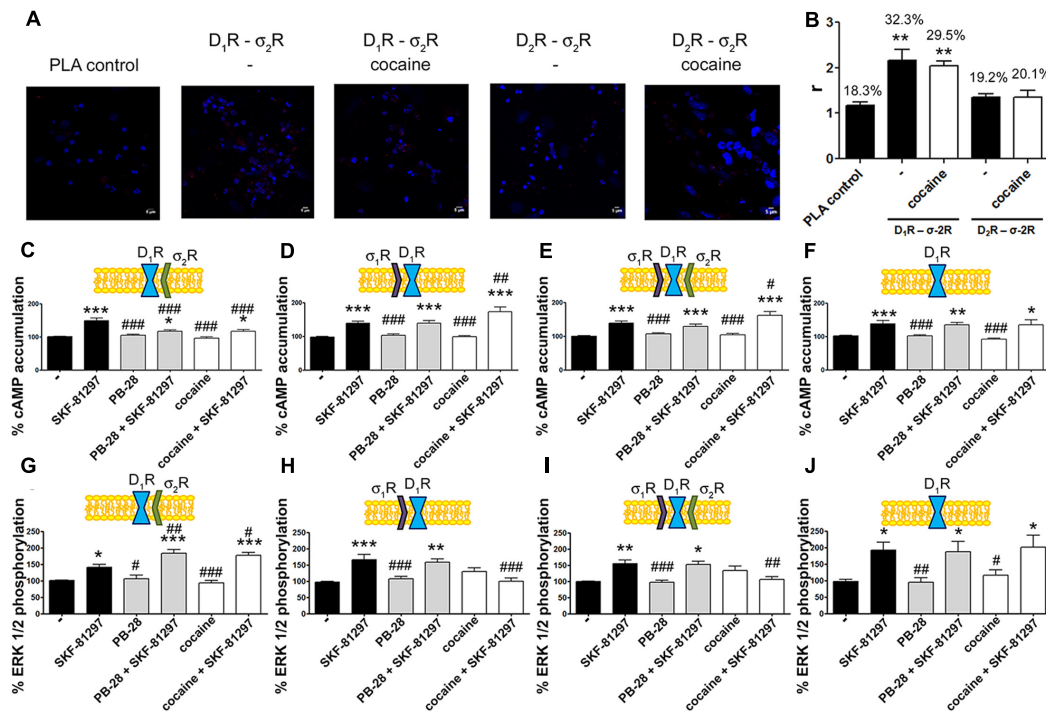
Schröder et al., 2009; Hamamoto et al., 2015), these results are similar to those obtained in cAMP read-outs. Another control was performed to show that pretreatment with the σ<sub>2</sub>R selective agonist, PB-28, did not result in any signal modulation in cells expressing D<sub>1</sub>R but silenced σ<sub>1</sub>R and σ<sub>2</sub>R expression (Figure 4H).

### σ<sub>2</sub>R Activation Blocks Dopamine D<sub>1</sub>R-Mediated Signaling in Primary Cultures of Striatal Neurons

A proximity ligation assay (PLA) was used to determine in primary cultures of striatal neurons whether D<sub>1</sub>R-σ<sub>2</sub>R complex expression was affected by cocaine pretreatment. Consequently, specific antibodies against D<sub>1</sub>R and σ<sub>2</sub>R were used in neurons treated or not with cocaine for 30 min (Figure 5A). 32% of cells showed punctuated staining (with 2.2 red spots/cell containing spots) surrounding Hoechst-stained nuclei (Figure 5B). These results indicate the occurrence of D<sub>1</sub>-σ<sub>2</sub> heteroreceptor complexes in striatal primary cultures of neurons. A control done in the absence of primary antibodies led to 18% of labeled cells (with 1.2 red spots/cell containing spots). The percentage of positive cells after a 30-min treatment with cocaine was around 30 (with 2 red spots/cell containing spots) (Figure 5B). Thus, cocaine pretreatment did not significantly

alter D<sub>1</sub>R-σ<sub>2</sub>R complex formation. When PLA was developed to detect D<sub>2</sub>R and σ<sub>2</sub>R complexes, the results (19% with 1.3 red spots/cell containing spots) were similar to those in the negative control (20% with 1.4 red spots/cell containing spots), i.e., no evidence of heteroreceptor formation was obtained. Pretreatment with cocaine did not lead to the appearance of heteromer complexes formed by D<sub>2</sub>R and σ<sub>2</sub>R (Figure 5B). These results agree with the BRET assays that did not find sign of interaction between the D<sub>2</sub>R-Rluc and σ<sub>2</sub>R-YFP but between the D<sub>1</sub>R-Rluc and σ<sub>2</sub>R-YFP pair.

To demonstrate the effect of cocaine over D<sub>1</sub>R-mediated signaling in a more physiological environment, we analyzed cAMP and MAP kinase signaling pathways in primary cultures of striatal neurons. As striatal neurons express the two sigma receptors, the siRNA approach was used to silence sigma receptor expression. On the one hand, in neurons transfected with siRNA for σ<sub>1</sub>R, and consequently expressing D<sub>1</sub>R and σ<sub>2</sub>R, cocaine and PB-28 led to a decrease in agonist-induced cAMP levels and to an enhancement in MAP kinase signaling (Figures 5C,G). On the other hand, in neurons transfected with the siRNA for σ<sub>2</sub>R, and consequently expressing D<sub>1</sub>R and σ<sub>1</sub>R, cocaine but not PB-28 induced an increase in the cAMP signal and a decrease in the ERK1/2 phosphorylation signal (Figures 5D,H). Most of these results agree with those obtained in the heterologous system. However, in striatal neurons



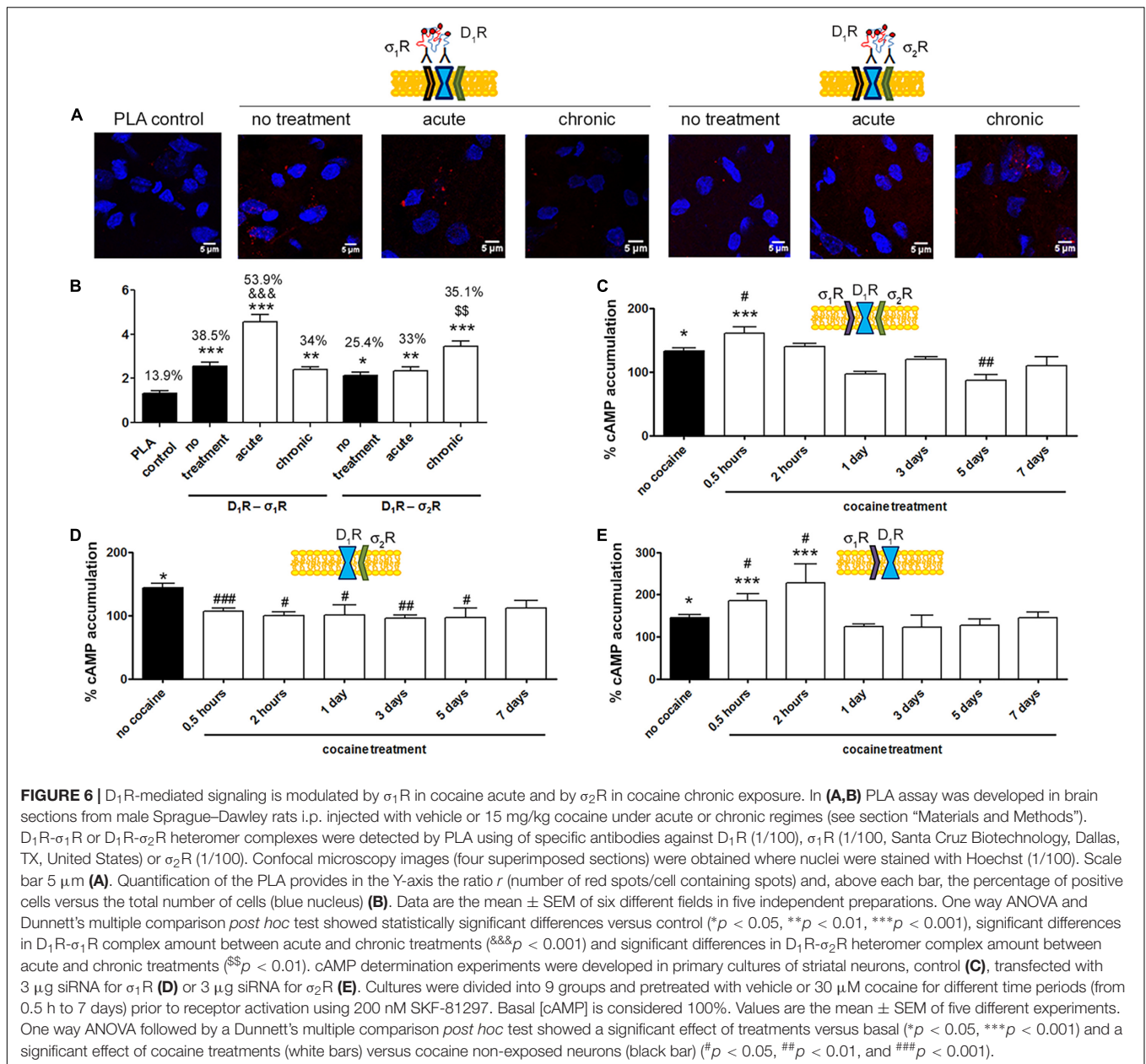
**FIGURE 5 |** Expression and function of  $\sigma_2R$ -D<sub>1</sub>R complexes in primary cultures of striatal neurons. In (A,B) PLA assay was developed in striatal primary cultures of neurons pretreated or not with cocaine 30  $\mu$ M for 30 min.  $\sigma_2R$ -D<sub>1</sub>R or  $\sigma_2R$ -D<sub>2</sub>R heteromer complexes were detected by the use of specific antibodies (1/100 dilution) against  $\sigma_2R$  and D<sub>1</sub>R or  $\sigma_2R$  and D<sub>2</sub>R. Confocal microscopy images (four superimposed sections) were obtained where nuclei were stained with Hoechst (1/100). Scale bar 5  $\mu$ m (A). Quantification of the PLA provides in the Y-axis the ratio *r* (number of red spots/cell containing spots) and, above each bar, the percentage of positive cells versus the total number of cells (blue nucleus) (B). Data are the mean  $\pm$  SEM of four different fields in five independent preparations. One way ANOVA and Dunnett's multiple comparison *post hoc* test showed statistically significant differences versus control (\*\* $p < 0.01$ ). Primary cultures of striatal neurons, control (E,I) or transfected with siRNA for  $\sigma_1R$  (C,G),  $\sigma_2R$  (D,H) or both (F,J) were treated with 30  $\mu$ M cocaine for 30 min or 300 nM PB-28 prior to 200 nM SKF-81297 stimulation. cAMP levels (C–F) and MAP kinase activation I (G–J) were determined. Basal [cAMP] is considered 100%. Values are the mean  $\pm$  SEM of 10–15 different experiments. One way ANOVA followed by a Dunnett's multiple comparison *post hoc* test showed a significant effect of treatments versus not treated cells (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) and a significant effect of treatments versus SKF-81297 (# $p < 0.05$ , ## $p < 0.01$ , and ### $p < 0.001$ ).

expressing D<sub>1</sub>R and both sigma receptors, cocaine treatment led to a net effect that showed predominance of  $\sigma_1R$ - versus  $\sigma_2R$ -mediated modulation (Figures 5E,I). These findings could be due to a higher expression of  $\sigma_1R$ -D<sub>1</sub>R complexes versus  $\sigma_2R$ -D<sub>1</sub>R but they may also result from the lower affinity of the cocaine/ $\sigma_2R$  binding (Lever et al., 2016). Finally, another control was performed to show that pretreatment with the  $\sigma_2R$  selective agonist, PB-28, or with cocaine, did not result in any signal modulation in cells expressing D<sub>1</sub>R but silenced  $\sigma_1R$  and  $\sigma_2R$  expression (Figures 5E,J).

### D<sub>1</sub>R-Mediated Signaling Is Modulated by $\sigma_1R$ in Acute and by $\sigma_2R$ in Chronic Conditions

*In situ* PLAs were performed to identify D<sub>1</sub>R- $\sigma_1R$  and D<sub>1</sub>R- $\sigma_2R$  heteroreceptor complexes in striatal sections from Sprague-Dawley rats receiving cocaine under acute or chronic regimes (see section “Materials and Methods”) (Figure 6A). When striatal sections of vehicle-treated animals were analyzed, it was observed that 38.5% of cells showed D<sub>1</sub>R- $\sigma_1R$  complexes

with 2.5 red spots/cell containing spots, while only 25% of cells showed D<sub>1</sub>R- $\sigma_2R$  complexes with 2.1 dots/cell (Figure 6B). When Sprague-Dawley rats were acutely treated with cocaine, it was observed that both D<sub>1</sub>R- $\sigma_1R$  and D<sub>1</sub>R- $\sigma_2R$  complex expression increased. However, the D<sub>1</sub>R- $\sigma_1R$  complexes doubled its expression while D<sub>1</sub>R- $\sigma_2R$  complex expression suffered a slight increase (respectively, 54% of cells showed red spots with 4.5 spots/cell and 33% with 2.3 spots/cell). Interestingly, in the case of rats chronically treated with cocaine, the D<sub>1</sub>R- $\sigma_1R$  heteromer complex expression was not affected (34% of cells showed red spots with 2.4 spots/cell containing spots) compared to control animals, while the D<sub>1</sub>R- $\sigma_2R$  heteromer expression significantly increased (35% of cells containing spots with 3.4 spots/cell containing spots) (Figure 6B). These results indicate that acute cocaine treatment strongly increases D<sub>1</sub>R- $\sigma_1R$  complexes formation in striatal rat sections but chronic cocaine treatment only drives D<sub>1</sub>R- $\sigma_2R$  complex expression. Then, we questioned if the cocaine-induced alterations in D<sub>1</sub>R- $\sigma_1R$  and D<sub>1</sub>R- $\sigma_2R$  complex expression had signaling consequences. To do so, we analyzed SKF-81297-induced cAMP production in primary cultures of striatal neurons pretreated with vehicle or cocaine for



different times (from 0.5 h to 7 days). Interestingly, we observed that at short times SKF-81297-induced cAMP levels were further increased. In agreement with results in HEK-293 cells, cocaine binding to the  $\sigma_1$ R induced a positive modulation over dopamine D<sub>1</sub>R-mediated signaling. When primary cultures of neurons were longer exposed to cocaine (1–7 days), SKF-81297-induced increase in cAMP levels was inhibited (Figure 6C). Taking into account the results in HEK-293 cells such effect seems associated to D<sub>1</sub>R- $\sigma_2$ R complex formation and to the ability of  $\sigma_2$ R to counteract the SKF-81297-induced increases of cAMP. To check whether these interpretations were correct, i.e., if sigma receptors were responsible of cocaine-induced modulations over D<sub>1</sub>R-mediated signaling, primary striatal neurons were transfected with siRNA specific for  $\sigma_1$ R or  $\sigma_2$ R. On the one hand, cocaine

pretreatment (0.5 h to 7 days) blocked SKF-81297-induced accumulation of cAMP levels in primary cultures of neurons transfected with siRNA for  $\sigma_1$ R, i.e., expressing D<sub>1</sub>R and  $\sigma_2$ R (Figure 6D). On the other hand, 0.5 h and 2 h pretreatment of cocaine potentiated the SKF-81297-induced increases in cAMP levels in primary neurons transfected with siRNA for  $\sigma_2$ R, i.e., expressing D<sub>1</sub>R and  $\sigma_1$ R. However, longer periods of cocaine exposure (1–7 days) produced no effect (Figure 6E). These results suggest that in acute cocaine treatment D<sub>1</sub>R form heteromers mainly with  $\sigma_1$ R, prevailing the D<sub>1</sub>R- $\sigma_1$ R-mediated signaling. In contrast, in the chronic situation, the increase of  $\sigma_1$ R-D<sub>1</sub> heteromer complex expression observed in acute conditions disappear but the increase in the D<sub>1</sub>R- $\sigma_2$ R complex expression is maintained, being the  $\sigma_2$ R responsible of the



cocaine modulation over D<sub>1</sub>R, hence prevailing the D<sub>1</sub>R-σ<sub>2</sub>R-mediated signaling.

## DISCUSSION

Sigma receptors are relevant in cocaine addiction, because binding of cocaine to these receptors modulates dopaminergic transmission. Although cocaine can bind to both σ<sub>1</sub>R and σ<sub>2</sub>R, they are not closely related and no common structural properties have been identified. With respect to the modulation of receptor-mediated signaling, a relevant difference is revealed by the formation of heteroreceptor complexes. On the one hand, σ<sub>1</sub>R interacts with D<sub>1</sub> and D<sub>2</sub> dopamine receptors (Navarro et al., 2010; Moreno et al., 2014). Interestingly, we here report that σ<sub>2</sub>R may form heteromeric complexes with D<sub>1</sub>R but not with D<sub>2</sub>R.

Information on PGRMC1/σ<sub>2</sub>R expression in brain is partial. Intlekofer and Petersen (2011) confirmed data by Krebs et al. (2000) showing enrichment of the receptor in nuclei of the hypothalamus that are important for female reproduction. Petersen et al. (2013) in 2013, reviewed neuroanatomical data on the expression PGRMC1 and related proteins in CNS neuroendocrine nuclei. To our knowledge, information on expression in other neural regions is either absent or preliminary. Interestingly, a recently developed fluorescent probe tested in rat brain indicates that the receptor is more present in neurons than in glial cells (Zeng et al., 2016). Despite good *in vitro* properties, some of the radiolabeled probes that were developed for *in vivo* σ<sub>2</sub> receptor imaging have not reached the final objective (Abate et al., 2013; Selivanova et al., 2015). In contrast, recently reported <sup>18</sup>F-labeled PET probes, with enhanced brain uptake and σ<sub>2</sub>R selectivity (in mice), show promise for *in vivo* imaging of the receptor in the human brain (Wang et al., 2017). Surely these novel tools will be instrumental to achieve a more detailed mapping of the receptor in the CNS, specially in those areas in which dopamine receptors are expressed. There is, however, strong evidence of expression in the striatum; for instance, a recent report shows that receptor agonist regulate dopaminergic input into the striatum and the receptor is presynaptically expressed the *nucleus accumbens* (Klawonn et al., 2017). Furthermore, pioneering studies by Werling and colleagues showed σ<sub>2</sub>R involvement in control of dopamine transporter activity in striatum (Derbez et al., 2002) and that the striatal receptor was a target of cocaine (Nuwayhid and Werling, 2006).

The results presented here and those already reported (Navarro et al., 2010, 2013) show that in equivalent experimental configurations, cocaine binding to σ<sub>1</sub>R improves the accumulation of cAMP mediated by D<sub>1</sub>R and inhibits MAP kinase signaling. Cocaine, via σ<sub>2</sub>R, blocks D<sub>1</sub>-mediated cAMP accumulation and enhances MAP kinase activation. Importantly, similar results were obtained in HEK-293T cells and primary neuronal cultures.

D<sub>1</sub>R can form complexes and high-order heteromers by interacting simultaneously with the σ<sub>1</sub> and σ<sub>2</sub> receptors, σ<sub>1</sub>R being able to displace σ<sub>2</sub>R, but not vice versa. Navarro et al. (2010)

reported an increase in the plasma membrane expression of σ<sub>1</sub>R after acute exposure to cocaine. When increase in σ<sub>1</sub>R levels in the plasma membrane occurs, σ<sub>2</sub>R is displaced from the D<sub>1</sub>R-σ<sub>2</sub>R or D<sub>1</sub>R-σ<sub>2</sub>R-σ<sub>1</sub>R heteroreceptor complexes. Such phenomenon results in increasing the amount of D<sub>1</sub>-σ<sub>1</sub> heteroreceptors and D<sub>1</sub>R signaling whereas, as reported by Navarro et al. (2013), reducing D<sub>2</sub>R-mediated actions. However, in a longer exposure to cocaine, the signaling mediated by the dopamine D<sub>1</sub>R fits more with that occurring via a D<sub>1</sub>R-σ<sub>2</sub>R functional unit. These data suggest that the initial cocaine-induced overexpression in the plasma membrane of the σ<sub>1</sub>R is transient; once these levels decrease, due to internalization or other still unknown mechanisms, σ<sub>2</sub> is the predominant receptor forming heteromers with dopamine D<sub>1</sub>R.

Motor control in the basal ganglia is achieved through a complex circuit composed of GABAergic neurons that contain mainly D<sub>1</sub>R (direct pathway) and GABAergic neurons containing mostly D<sub>2</sub>R (indirect pathway) (Grillner and Robertson, 2016). Fine motor control is achieved by a balance of dopaminergic signals, one via D<sub>1</sub> receptors, which are G<sub>s</sub> coupled, and another via D<sub>2</sub> receptors, which are G<sub>i</sub> coupled (Jenner, 1995; Gerfen, 2000). The deterioration of motor control by cocaine depends on the imbalance of the direct/indirect pathway, but the underlying mechanism remains unclear. Although the scenario is complex, cocaine is known to increase cAMP levels in cells expressing D<sub>1</sub>R-σ<sub>1</sub>R (Navarro et al., 2010). Therefore, cocaine seems to be increasing in the direct pathway the cAMP-dependent dopaminergic output, namely activation of protein kinase A and cAMP-regulated DARPP-32 phosphoprotein (Svenningsson et al., 2004). Through the same receptor (σ<sub>1</sub>R), cocaine leads to a deterioration of the dopaminergic performance of the indirect route (Navarro et al., 2013). In addition to the imbalance resulting from these σ<sub>1</sub>R-dependent effects, our results demonstrate that trimers of D<sub>1</sub>, σ<sub>1</sub>, and σ<sub>2</sub> receptors may be formed and that cocaine acting on these heteromers reduces the negative modulation exerted by the D<sub>1</sub>R-σ<sub>1</sub>R complexes.

The results here presented also show that the MAP kinase signaling pathway is particularly affected by the action of cocaine upon dopamine-sigma heteroreceptors. While in cells expressing the D<sub>1</sub>R-σ<sub>1</sub>R heteromer, cocaine decreased ERK1/2 phosphorylation, cocaine did the opposite in cells expressing the D<sub>1</sub>R-σ<sub>2</sub>R heteromer. It is known that ERKs are involved in the plastic changes induced by the consumption of drugs of abuse (Radwanska et al., 2005). In addition, the inhibition of ERK phosphorylation alters learned place-preference in a paradigm of drug-of-abuse consumption, whereas activation of ERK1/2 is necessary to establish the association between place preference and drug consumption (Valjent et al., 2006; Du et al., 2017). In this context, knocking down ERK1 has shown that enhanced ERK2 signaling and repeated exposure to the drug facilitate the plastic changes leading to drug addiction (Ferguson et al., 2006). It should be noted that the temporal pattern of MAP kinase activation in the mouse brain is differently induced by addictive or non-addictive drugs (Valjent et al., 2004). Interestingly, Zhang et al. (2017) have described that D<sub>1</sub> receptor antagonists alter in cocaine-treated mice the length of *nucleus accumbens* postsynaptic densities, i.e., cocaine-induced

long-term plasticity; however, the mechanism underlying this phenomenon has not been described. According to the previous reports and to our results, it may be suggested that potentiation of MAP kinase pathway mediated by the  $D_1R$ - $\sigma_2R$  heteromer may be the mechanism by which the  $\sigma_2R$  would induce long term neuronal plasticity. The predominant role of the  $\sigma_1R$  in acute cocaine use shifts to a more relevant role of  $\sigma_2R$  in the chronic condition leading to the establishment of addiction. In any case, the relative expression of the two receptors in a given neuron seems important in determining the fate of the cell when the drug of abuse is consumed.

In acute cocaine exposure,  $\sigma_1R$  modulation of  $D_1R$ -mediated signaling prevails, but in longer exposures, there is a shift to regulation by  $\sigma_2R$ . Recently, Singer et al. (2017) have determined that neuronal plasticity initiate 2 h after cocaine exposure. The mechanism of action described in this paper cannot explain some of the results reported by Matsumoto et al. (2007) and Lever et al. (2014) who report that  $\sigma_2R$  receptor antagonists block the effects of cocaine-induced hyperlocomotion. It should be, however, noted that a recent report show benefits of a  $\sigma_2R$  -selective agonist, siramesine, for decreasing cocaine effects via reduction of dopaminergic and glutamatergic input to the striatum (Klawonn et al., 2017). On the one hand, it is a reasonable assumption that  $\sigma_1R$  is more involved in the regulation of  $D_1R$  signaling at acute exposure. However, it remains to be determined whether some of the results reported on the impact of cocaine on locomotion are due to the use of non-selective ligands, that is, ligands that can bind to both sigma receptors and alter their function. Alternatively, it may happen that  $\sigma_2R$  is also affecting the direct route in acute conditions. What our results undoubtedly indicate is that  $\sigma_2R$  becomes the main player in conditions of chronic exposure to the drug. In summary, some of the addictive and motor actions of cocaine are the result of a balance between cocaine- $\sigma_1R$  versus cocaine- $\sigma_2R$  impact on activation of  $D_1R$  and  $D_2R$  (and  $D_1R$ - $D_2R$ , see Perreault et al., 2016) in *ad hoc* CNS circuits.

## MATERIALS AND METHODS

### Reagents

Cocaine-chlorhydrate was provided by the Spanish *Agencia del Medicamento* (Ref. n°: 2003C00220).  $\sigma_2R$  agonist, 1-Cyclohexyl-4-[3-(1,2,3,4-tetrahydro-5-methoxy-1-naphthalenyl)propyl]piperazine dihydrochloride (PB-28),  $D_1R$  agonist ( $\pm$ )-6-Chloro-2,3,4,5-tetrahydro-1-phenyl-1H-3-benzazepine hydrobromide (SKF-81297) and  $D_2R$  agonist, sumanirole, were purchased from Tocris, Bristol, United Kingdom.

### Fusion Proteins and Expression Vectors

cDNAs for human versions of  $D_1R$ ,  $D_2R$ ,  $\sigma_1R$ , or  $\sigma_2R$  cloned into pcDNA3.1, were amplified without their stop codons using sense and antisense primers harboring: *EcoRI* and *KpnI* sites to subclone  $D_1R$ ,  $D_2R$ ,  $\sigma_1R$ , and  $\sigma_2R$  in pcDNA3.1Rluc vector (pRluc-N1, PerkinElmer Life and Analytical Sciences, Wellesley, MA, United States) or *HindIII* and *BamHI* sites to clone  $D_1R$ ,

$D_2R$ ,  $\sigma_1R$ , and  $\sigma_2R$  in pEYFP-N1 vector (enhanced yellow variant of GFP; Clontech), or *EcoRI* and *BamHI* sites to clone  $\sigma_2R$  in a cherry-containing vector (pcDNA3.1Cherry). Amplified fragments were subcloned to be in-frame with restriction sites for pRluc-N1, pEYFP-N1, or pcDNA3.1Cherry vectors to provide plasmids that express proteins fused to *Renilla* Luciferase ( $D_1R$ -Rluc,  $D_2R$ -Rluc,  $\sigma_1R$ -Rluc, and  $\sigma_2R$ -Rluc), YFP ( $D_1R$ -YFP,  $D_2R$ -YFP,  $\sigma_1R$ -YFP, and  $\sigma_2R$ -YFP) or cherry ( $\sigma_2R$ -Cherry) at the C-terminal end.

### Cell Lines and Transient Transfection

HEK-293T human embryonic kidney cells were grown at 37°C in a humid atmosphere with 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fischer Scientific, Madrid, Spain) supplemented with 2 mM L-glutamine, 100  $\mu$ l/ml sodium pyruvate, 100 U/ml penicillin/streptomycin, MEM Non-Essential Amino Acid Solution (1/100) and 5% (v/v) heat inactivated foetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, United Kingdom). Cells were transiently transfected with constructs encoding for receptors, fusion proteins, and/or siRNAs by the polyethylenimine (PEI; Sigma-Aldrich, St. Louis, MO, United States) method. Transfected cells were incubated in serum-free medium that after 4 h was replaced by complete medium. Experiments were carried out 48 h later.

### Neuronal Primary Cultures

Primary cultures of striatal neurons were obtained from 19-day embryos of Sprague-Dawley rats. Cells were isolated as described in Hradsky et al. (2013) and plated at a confluence of 40,000 cells/0.32 cm<sup>2</sup>. Cells were maintained for 12 days in Neurobasal medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 2% (v/v) B27 supplement (Gibco) in 6-well plates. When indicated, cells were transiently transfected with the corresponding siRNA (3  $\mu$ g plasmid siRNA per well) using the Lipofectamine<sup>TM</sup> 2000 (Invitrogen, Life Technologies, Darmstadt, Germany). Transfected cells were incubated in serum-free medium that after 4 h was replaced by complete medium. Experiments were carried out 48 h later.

### Cocaine Treatment of Sprague-Dawley Rats

Male Sprague-Dawley rats weighing 200–220 g were selected for the experiments. Rats were kept in controlled environment with 12 h light-dark cycle at 21°C room temperature. Food and water were provided *ad libitum*. Experimental procedures were approved by the Bioethical Committee of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile and follow the international guidelines (NIH Guide for the Care and Use of Laboratory Animals). Rats were housed and handled in colony for three days, and then were divided in two experimental groups: acute and chronic, with respective saline controls. Chronic cocaine administration consisted in two injections of cocaine (15 mg/kg, i.p.) per day for 14 days at 11:00 A.M. and 5:00 P.M., as described by Liu et al. (2005). Acute cocaine administration consisted of two injections of

cocaine (15 mg/kg, i.p.) for only one day. The same protocol of administration was used in control animals receiving saline injections. Rats were sacrificed 17 h. after the last saline or cocaine injection following the protocol of Liu et al. (2005). Cocaine HCl was donated by the National Institute on Drug Abuse (NIDA, United States).

## Immunocytochemistry

HEK-293T cells were treated with 30  $\mu$ M cocaine or vehicle for 30 min, then were washed with PBS, fixed in 4% paraformaldehyde for 15 min and washed with PBS containing 20 mM glycine to quench free aldehyde groups. After permeabilization with PBS-glycine buffer containing 0.2% Triton X-100 for 5 min, cells were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h at room temperature. D<sub>1</sub>R-YFP and D<sub>2</sub>R-YFP were detected by its own fluorescence (wavelength 530 nm), and  $\sigma_2$ R-Rluc was stained using a primary anti-Rluc mouse monoclonal antibody (1/200, Millipore, CA, United States) for 1 h, washed and stained for another hour with the secondary Cy3-conjugated donkey anti-mouse antibody (1/200, Jackson ImmunoResearch Laboratories, West Grove, PA, United States). Nuclei were stained with Hoechst (1/100, Sigma-Aldrich, St. Louis, MO, United States) and then samples were rinsed several times and mounted with Mowiol 30% (Calbiochem). Images were taken using a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany).

## Proximity Ligation Assay

For PLAs, HEK-293T cells or primary cultures of striatal neurons were grown on glass coverslips while cocaine-administered rat sections were mounted and dried directly on glass coverslips. Samples were treated with 30  $\mu$ M cocaine or vehicle for 30 min, then were washed with PBS and fixed in 4% paraformaldehyde for 15 min, washed with PBS containing 20 mM glycine, permeabilized with the same buffer containing 0.05% Triton X-100 for 5 min, and washed with PBS. Then, samples were incubated at 37°C with the blocking solution for 1 h. Heteromers were detected using the Duolink *in situ* PLA detection kit (OLink Bioscience, Bioscience, Uppsala, Sweden) following the instructions of the supplier. To detect D<sub>1</sub>R- $\sigma_2$ R or D<sub>2</sub>R- $\sigma_2$ R heteromers, cells and primary cultures were incubated overnight with anti D<sub>1</sub>R (1/100), anti  $\sigma_2$ R (1/100) and Hoechst (1/100), or anti D<sub>2</sub>R (1/100), anti  $\sigma_2$ R and Hoechst. Samples were processed using PLA probes that bind to the primary antibodies (Duolink II PLA probe anti-mouse plus and Duolink II PLA probe anti-goat minus). Images were taken using a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an apochromatic 63 $\times$  oil-immersion objective (numerical aperture 1.4) and 405 and 561 nm laser lines. For each field of view a stack of two channels (one per staining) and 4–6 Z stacks with a step size of 1  $\mu$ m were acquired. Quantification of the number of cells containing one or more red spots versus total cells (blue nuclei) and, in cells containing spots, of the number of red spots/cell ratio, was conducted using dedicated software known as Duolink ImageTool (ref: DUO90806, Sigma-Olink). This software has been developed for quantification of PLA signals and cell nuclei in images

generated from fluorescence microscopy. One-way ANOVA followed by Dunnett's *post hoc* multiple comparison test was used for statistical analysis.

## Resonance Energy Transfer

For bioluminescence resonance energy transfer (BRET), HEK-293T cells were transiently cotransfected with a constant amount of cDNA encoding for proteins fused to Rluc and increasing amounts of cDNAs corresponding to proteins fused to YFP (see figure legends). To normalize the number of cells, protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using BSA dilutions as standards. To quantify protein YFP expression, cells (20  $\mu$ g of protein) were distributed in 96-well plates (black plates with a transparent bottom), and fluorescence was read in the FluoStar Optima Fluorimeter (BMG Labtech, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10-nm bandwidth excitation filter at 400 nm reading. Protein fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing the BRET donor alone. For BRET measurements, the equivalent of 20  $\mu$ g of cell suspension was distributed in 96-well plates (Corning 3600, white plates; Sigma), and coelenterazine H (5  $\mu$ M; Invitrogen) was added. After 1 min, readings were obtained using a Mithras LB 940 (Berthold Technologies), which 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 protein-Rluc luminescence, readings were also performed 10 min after addition of coelenterazine H. For SRET assays, cells were transiently cotransfected with constant amounts of cDNA encoding for both receptor fused to Rluc and YFP proteins, and with increasing amounts of cDNA corresponding to the receptor fused to cherry protein. After 48 h of transfection, quantification was performed in parallel in aliquots of transfected cells (20  $\mu$ g of protein): quantification of receptor YFP or receptor Rluc expression was performed as indicated for BRET experiments. Quantification of receptor-Cherry expression, cells were distributed in 96-well plates (Corning black plates with a transparent bottom), and fluorescence was read in the FluoStar Optima Fluorimeter using a 10 nm bandwidth excitation filter at 590 nm reading. For SRET quantification, cells were distributed in 96-well plates (black plates with transparent bottom), and coelenterazine H (5  $\mu$ M) was added. After 1 min, the readings were collected using a FluoStar Optima Fluorimeter, which allows the integration of the signals detected in the short-wavelength filter at 530 nm and the long-wavelength filter at 590 nm. Net BRET and net SRET were defined as [(long-wavelength emission)/(short-wavelength emission)] – C<sub>f</sub>, where C<sub>f</sub> corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the Rluc construct expressed alone in the same experiment. Both fluorescence and luminescence were measured before every experiment to confirm similar donor expressions (~100,000 bioluminescence units) while monitoring the increase in acceptor expression (1,000–40,000 fluorescence units). BRET or SRET was expressed as, respectively, millibRET (mBU) or milliSRET (mSU) units (net BRET or SRET  $\times$  1,000). Data were fitted to a nonlinear

regression equation, assuming a single-phase saturation curve with GraphPad Prism software (GraphPad Software). The relative amount of BRET or SRET is given as a function of  $100 \times$  the ratio between the fluorescence of the acceptor (YFP or cherry) and the luciferase activity of the donor (Rluc).

## cAMP Determination

cAMP levels were assayed with different forskolin concentrations and cell densities to select the most appropriate conditions of the assay, which were  $0.5 \mu\text{M}$  forskolin and 5,000 HEK-293T cells or 7,500 neurons. Transfected HEK-293T cells or neurons were incubated in serum-free medium for 3 h before the experiment. Then, cells were placed in 384-well microplates in medium containing  $50 \mu\text{M}$  zardaverine (Tocris Bioscience). Cells were then preincubated with vehicle, the  $\sigma_2\text{R}$  agonist, PB-28 ( $300 \text{ nM}$ ) or cocaine ( $30 \mu\text{M}$ ) for 15 min, followed by dopaminergic stimulation with the  $\text{D}_1\text{R}$  agonist, SKF-81297 ( $200 \text{ nM}$ ), the  $\text{D}_2\text{R}$  agonist, sumanirole ( $500 \text{ nM}$ ) or vehicle. After another incubation period of 15 min,  $0.5 \mu\text{M}$  forskolin or vehicle were added. Readings were performed 15 min later by the use of a homogeneous time-resolved fluorescence energy transfer (HTRF) method requiring the Lance Ultra cAMP kit (PerkinElmer) and fluorescence readings (at  $635 \text{ nm}$ ) in a PHERAstar Flagship microplate equipped with a time-resolved fluorescence optical module (BMG Labtech).

## ERK1/2 Phosphorylation

To determine ERK1/2 phosphorylation, 40,000 HEK-293T cells/well or 50,000 neurons/well were plated in transparent Deltalab 96-well plates and kept in the incubator for 48 h. The medium was substituted by serum-free DMEM medium for 2–4 h before initiating the experiment. Then, HEK-293T cells and striatal neurons were pretreated for 10 min at  $25^\circ\text{C}$  with vehicle, PB-28 ( $300 \text{ nM}$ ) or cocaine ( $30 \mu\text{M}$ ) followed by the addition of  $200 \text{ nM}$  SKF-81297, the  $\text{D}_1\text{R}$  specific agonist. 10 min after activation, cells/neurons were placed on ice and washed twice with cold PBS before the addition of  $30 \mu\text{l}$  of lysis buffer for 15 min. Supernatants ( $10 \mu\text{l}$ ) were placed in white ProxiPlate 384-well microplates, and ERK1/2 phosphorylation was determined using the AlphaScreen<sup>®</sup>SureFire<sup>®</sup> kit (Perkin Elmer) and the EnSpire<sup>®</sup> Multimode Plate Reader (PerkinElmer, Waltham, MA, United States).

## Label-Free Dynamic Mass Redistribution Assays (DMR)

HEK-293T cells and neuronal primary cultures were seeded in 384-well sensor microplates for 24 h before the assay to obtain 70–80% confluent monolayers constituted by 5,000

HEK-293T cells or 14,000 neurons per well. Previous to the assay, cells were washed twice with assay buffer (HBSS with  $20 \text{ mM}$  HEPES and  $0.1\%$  DMSO,  $\text{pH}$  7.15) and incubated for 2 h in  $40 \mu\text{l}$ /well of assay-buffer in the reader at  $24^\circ\text{C}$ . Hereafter, the sensor plate was scanned and a baseline optical signature was recorded before adding ( $10 \mu\text{l}$ ) vehicle, cocaine ( $30 \mu\text{M}$ ) or PB-28 ( $300 \text{ nM}$ ) for 30 min followed by SKF-81297 ( $200 \text{ nM}$ ) addition. All compounds dissolved in assay buffer. Then, DMR responses were monitored for at least 3,600 s using an EnSpire<sup>®</sup> Multimode Plate Reader (PerkinElmer Life and Analytical Sciences, Waltham, MA, United States). Sensitive measurements of changes in local optical density mimicking cellular mass movements induced upon receptor activation were detected using EnSpire Workstation Software v4.10, and curves were normalized with respect to the baseline.

## Data Analysis

The data in graphs are the mean  $\pm$  SEM. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Parametric statistic methods were used, because results in the different groups showed normality and homogeneity of variance. Significance was analyzed by one-way ANOVA, followed by Dunnett's multiple comparison *post hoc* test. GraphPad Prism software version 5 was used for the statistical analysis. Significant differences were considered when  $p < 0.05$ .

## AUTHOR CONTRIBUTIONS

GN, EC, and RF designed the experiments and directed the project. DA did many of the cell and molecular assays and did the statistics of the results in the laboratory of the University of Barcelona. KG designed the experiments to obtain cocaine-treated animals in the laboratory in Chile. MM and IV-Q administered drugs to animals, prepared the brain sections, and performed the immunological-based histochemical assays. GN and RF wrote the first draft of the manuscript, which was further edited by DA, KG, EC, and IV-Q.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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