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Invited review

Differential effect of amphetamine over the corticotropin-releasing factor CRF_2 receptor, the orexin OX_1 receptor and the CRF_2-OX_1 heteroreceptor complex

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ABSTRACT

Stress is one of the factors underlying drug seeking behavior that often goes in parallel with loss of appetite. We here demonstrate that orexin 1 receptors (OX_1R) may form heteromeric complexes with the corticotropin releasing factor CRF_2 receptor. Two specific features of the heteromer were a cross-antagonism and a blockade by CRF_2 of OX_1R signaling. In cells expressing one of the receptors, agonist-mediated signal transduction mechanisms were potentiated by amphetamine. Sigma 1 (σ_1) and 2 (σ_2) receptors are targets of drugs of abuse and, despite sharing a similar name, the two receptors are structurally unrelated and their physiological role is not known. We here show that σ_1 receptors interact with CRF_2 receptors and that σ_2 receptors interact with OX_1R. Moreover, we show that amphetamine effect on CRF_2 receptors was mediated by σ_1R whereas the effect on OX_1 receptors was mediated by σ_2R. Amphetamine did potentiate the negative cross-talk occurring within the CRF_2-OX_1 receptor heteromer context, likely by a heteroreceptor complex involving the two sigma receptors and the two GPCRs. Finally, *in vivo* microdialysis experiments showed that amphetamine potentiated orexin A-induced dopamine and glutamate release in the *ventral tegmental area* (VTA). Remarkably, the *in vivo* orexin A effects were blocked by a selective CRF_2R antagonist. These results show that amphetamine impacts on the OX_1R-, CRF_2R- and OX_1R/CRF_2R-mediated signaling and that cross-antagonism is a unique tool for *in vivo* detection of GPCR heteromers.

1. Introduction

The first reference to a possible involvement of orexins in the effects of drugs of abuse appeared in 2003 and showed that orexigenic peptide neurons respond to the chronic administration of morphine followed by abrupt withdrawal symptoms caused by opioid receptor antagonists (Georgescu et al., 2003). Subsequent studies examined the effect of orexigenic peptides in the reward mechanism associated with consumption of drugs of abuse. It is today accepted that orexins have an important role in responses associated with both food and drug rewards (Aston-Jones et al., 2010; Harris et al., 2005). The exact nature of the effects of orexins in this context is unknown because of their complex involvement in different aspects of drug addiction, aversive or appetitive motivation, interactions with Pavlovian or instrumental learning processes and induced hedonic states.

Stress and drug addiction correlate in advanced societies. Therefore, the mechanisms of stress and of reward must be interconnected. A relevant regulator of stress responses is the corticotropin releasing factor (CRF), which is one of the main players in the hypothalamic–pituitary–adrenal (HPA) axis (Gallagher et al., 2008). CRF is a peptide synthesized and secreted by hypophysiotropic neurons that, in response to stress, is released into the pituitary portal veins that give access to the anterior pituitary gland. Therefore, CRF activates its cognate receptors

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and induces the release of the adrenocorticotropic hormone (ACTH) into the systemic circulation, where it travels to its main target, the adrenal gland. ACTH stimulates the synthesis and secretion of glucocorticoids in the adrenal cortex (Bamberger et al., 1996). In fact, the actions of glucocorticoids are mediated by glucocorticoid (GR) and mineral corticoid (MR) receptors, that are widely expressed in the brain, including areas involved in emotion, memory, and behavior such as the septum, hippocampus, and prefrontal cortex (Morimoto et al., 1996; Viengchareun et al., 2007).

A first aim of this paper was to investigate whether a drug of addiction, namely amphetamine, affected the response of orexin or of CRF receptors in a heterologous system. As orexin and CRF receptors belong to the superfamily of G-protein-coupled receptors (GPCR), G-protein-dependent and –independent signaling pathways were analyzed. As GPCRs often form heteromeric complexes, the potential of receptors to form direct receptor-receptor interactions was assayed and proved.

Sigma 1 receptors (σ_1R) are an atypical type of membrane receptors whose exact function is not known. They have been proposed as mediators of pluripotent modulation in living cells (Su et al., 2016) and research on this protein is gaining momentum due to its potential as target to combat neuropathic pain (Corbera et al., 2006; Mei and Pasternak, 2002; Sun et al., 2016). In fact, the three-dimensional structure has been recently elucidated. σ_1R protomers, with a single transmembrane domain and a C-terminal tail having a cupin-like β-barrel with a buried ligand-binding site, arranged into homotrimers (Schmidt et al., 2016). Interestingly, it is a target of different drugs of abuse, cocaine and methamphetamine among them (Cobos et al., 2008; Nguyen et al., 2005). Due to the reports showing that σ_1R may interact with metabotropic receptors for a variety of hormones/neurotransmitters, it was tempting to speculate that the receptor may be regulating the expression and function of GPCRs and of GPCR heteromers. As σ_1R may form heteromers with corticotropin-releasing factor CRF_1 receptor (Navarro et al., 2015), the occurrence of σ_1R-containing heteroreceptor complexes and the effect of amphetamine on orexin-CRF heteromeric-mediated signaling was also approached using *in vitro* experiments.

2. Results

2.1. Orexin-1 receptors form heteromeric complexes with CRF_2 receptors in a heterologous expression system

To determine whether orexin receptor 1 (OX_1R) colocalized at the plasma membrane level with corticotropin-releasing factor 2 receptor (CRF_2R), immunocytochemistry assays were undertaken in HEK-293T cells transfected with 0.75 μg cDNA for CRF_2R-YFP, 0.4 μg cDNA for OX_1R-RLuc or both. CRF_2R expression was detected by the YFP own fluorescence while OX_1R expression was detected by a specific antibody against RLuc (1/100) followed by a secondary Cy3-antibody. OX_1R showed a membrane localization and CRF_2R was localized at the plasma membrane but also in intracellular structures (Fig. 1A). Colocalization of both receptors was marked (detected in yellow in Fig. 1).

Immunocytochemistry assays are not suitable to demonstrate physical interactions. Thus, bioluminescence energy transfer (BRET) assays were developed in HEK-293T cells expressing a constant amount of OX_1R-RLuc and increasing amounts of CRF_2R-YFP. The saturation BRET curve shown in Fig. 1B indicates a specific interaction between CRF_2R and OX_1R ($BRET_{max}$ 46 ± 3 mBU, $BRET_{50}$ 33 ± 6) (Fig. 1B). As negative control, HEK-293T cells were transfected with the cDNA for GHS-R1a-RLuc (0.75 μg cDNA) and increasing amounts of the cDNA for CRF_2R-YFP (0.1–1.2 μg cDNA) and an unspecific linear signal was obtained (Fig. 1B).

2.2. Functional characterization of CRF_2R-OX_1R heteromeric complexes

It is well established that GPCR heteroreceptor complexes display unique properties that differ from those of the individual receptors. To describe the heteromer-specific signaling characteristics, HEK-293T cells coexpressing CRF_2R and OX_1R were treated with the specific antagonist for CRF_2R, K41498 (1 μM), the specific antagonist for OX_1R, SB334867 (1 μM) or vehicle, followed by activation of receptors with CRF (100 nM) and/or Orexin A (100 nM). Four different techniques were used to address signal transduction. In cAMP determination assays we observed that CRF treatment induced a significant increase in cAMP levels, i.e. Gs-coupling of the CRF_2R. Orexin A decreased 0.5 μM

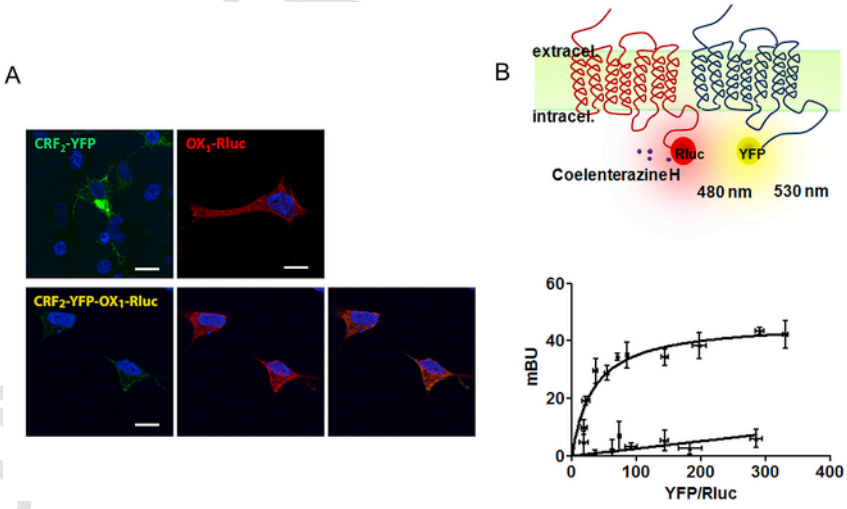

Fig. 1. Expression and interaction of CRF_2 and orexin OX_1 receptors in a heterologous system. Panel A: Receptors expressed in HEK-293T cells transfected with 0.75 μg cDNA for CRF_2-YFP, 0.4 μg cDNA for OX_1-RLuc or both were identified by YFP fluorescence (green) or by a monoclonal anti-RLuc (1/100) primary antibody and a cyanine-3-conjugated (1/200) secondary antibody (red). Colocalization of both receptors is shown in yellow. Nuclei were stained with Hoechst (1/100, blue). Relative fluorescence intensity was 59 for CRF_2-YFP and 46 for OX_1-Rluc (SD < 10%). Scale bar 20 μm. Panel B: BRET was performed in HEK-293T cells expressing a constant amount of OX_1R-RLuc (0.4 μg cDNA) (or GHS-R_{1a}-RLuc −0.75 μg cDNA-as negative control) and increasing amounts of CRF_2R-YFP (0.1–1.2 μg cDNA) (Fig. 1B). Values are the mean ± SEM of 6 different experiments. A schematic representation of the assay is shown (top). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

forskolin-induced cAMP levels, thus underscoring Gi-coupling of OX_1R (Fig. 2A). Interestingly, coactivation with CRF and Orexin A induced a signal similar to that obtained with CRF, indicating that CRF_2R engagement blocks OX_1R coupling to Gi in the CRF_2R-OX_1R complex. When cells were pretreated with K41498, the CRF_2R antagonist, a complete inhibition of both the CRF and the Orexin A effects was observed. Such cross-antagonism is an often-found property of GPCR heteromers (Franco et al., 2007). The pretreatment with the antagonist of OX_1R, SB334867, offered a partial cross-antagonism when cells were stimulated with CRF (Fig. 2A). In the analysis of calcium mobilization, CRF induced no effect whereas Orexin A, consistent with canonical Gq coupling of OX_1R, produced a transient response with a maximum of cytosolic calcium concentration at 30 s after ligand addition. Interestingly, coactivation with both ligands significantly reduced Orexin A-induced effects. Moreover, cross-antagonism was found; in fact, not only SB334867 but also K41498 inhibited the effect of Orexin A (Fig. 2B). In ERK1/2 phosphorylation and label-free assays, the effect of either CRF or Orexin A was significantly higher than that obtained when cells were simultaneously treated with the two agonists (Fig. 2C and D). The results reflect allosteric interactions within the heteromer that impair signaling when the two protomers are activated. Once more, a cross-antagonism phenomenon was detected as shown by the effect of antagonists displayed in Fig. 2C and D. Taken together, the functional characterization of the CRF_2R-OX_1R complex results in finding a partial or total cross-antagonism and an allosteric-driven negative cross-talk that was more evident when quantifying the effect of Orexin A in the presence of CRF.

2.3. Amphetamine potentiates CRF_2 and OX_1 receptor signaling in a heterologous expression system

As the sensation of hunger is suppressed by amphetamine, we investigated whether the drug could affect CRF_2-OX_1 receptor heteromer-mediated signaling. HEK-293T cells transfected with the cDNA (0.6 μg) for CRF_2R were pretreated for 30 min with amphetamine (2 μM) (white bars) or vehicle (black bars) and activated using CRF (100 nM) (Fig. 3A and B). The increase in cAMP levels (around 290%) was further enhanced by the drug (390%). Due to the lack of OX_1R expression, coactivation with CRF and Orexin A provided in both conditions, with and without amphetamine, similar results as with CRF (Fig. 3A). In OX_1R-expressing cells, Orexin A led to a reduction in the forskolin-induced cAMP levels (24% reduction), that was potentiated (to 48% reduction) when 2 μM amphetamine was added (Fig. 3B). Interestingly, similar results were obtained when CRF_2R or OX_1R expressing cells (0.6 μg cDNA each) were analyzed by the DMR label-free assay (Fig. 3 C, D), i.e. a significant increase in agonist-induced changes in cell mass density was found in cells pretreated with amphetamine (2 μM) for 30 min. In agreement with its coupling to a Gs protein, activation of the receptor in CRF_2R expressing HEK-293T cells did not led to alteration in cytoplasmic calcium levels (Fig. 3E). In OX_1R-expressing cells, Orexin A produced a characteristic peak of cytosolic calcium that was further increased with amphetamine (2 μM) pretreatment. These results agree with those observed in cAMP and DMR signals, indicating that amphetamine pretreatment potentiates both CRF_2R and OX_1R G-protein-dependent signaling pathways. Finally, the MAPK activation assays showed that treatment with 100 nM CRF in cells individually expressing CRF_2R increased by 2-fold the basal levels of ERK1/2 phosphorylation and amphetamine pretreatment led to a relatively small potentiation (Fig. 3G). In contrast, in cells expressing OX_1R, the significant effect of 100 nM Orexin A (>4-fold increase over basal) was significantly reduced upon 2 μM amphetamine pretreatment (Fig. 3H). These results indicate that amphetamine impacts on both CRF_2R- and OX_1R-mediated dependent signaling.

2.4. Amphetamine effects over CRF_2R depend on σ_1R expression

Corticotropin-releasing hormones act via two different receptors, CRF_1R and CRF_2R. We have recently reported (Navarro et al., 2015) the physical interaction between CRF_1R and σ_1R, demonstrating that CRF_1R receptors participate in cocaine-dependent actions. It has also been reported (Cobos et al., 2008; Nguyen et al., 2005) that different drugs of abuse, cocaine and methamphetamine among them, may bind

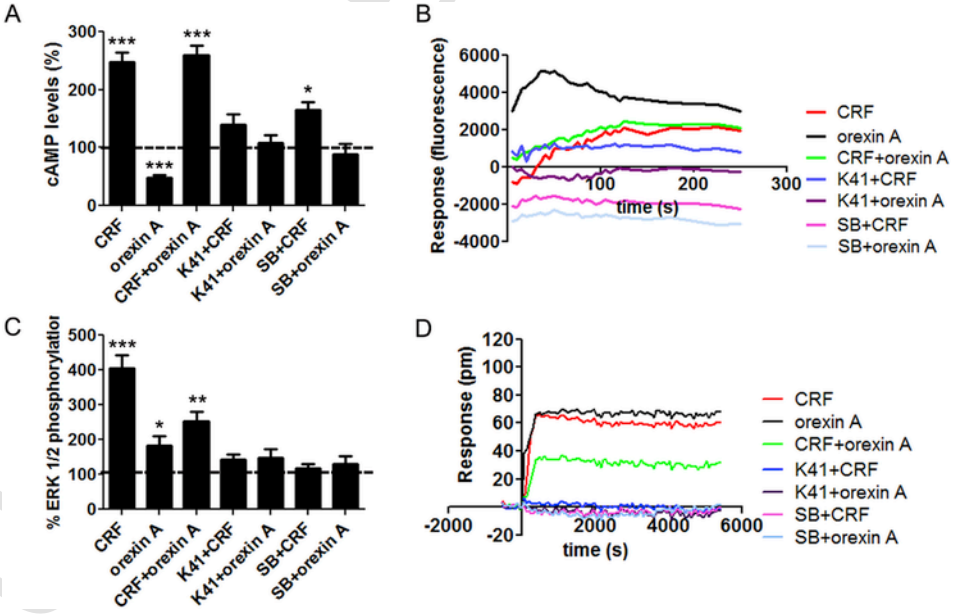

Fig. 2. Functional characterization of the CRF_2-OX_1 heteroreceptor complex. HEK-293T cells transfected with 0.5 μg cDNA for CRF_2 receptor and with 0.6 μg cDNA for OX_1 receptor were pretreated with the CRF_2 receptor antagonist, K41498 (1 μM) or the OX_1 receptor antagonist SB334687 (1 μM) followed by treatment with CRF (100 nM), orexin A (100 nM) or both. cAMP levels (A), calcium release (B), ERK1/2 phosphorylation (C) and DMR (D) data were collected. In panel A data are represented in percentage (100% represented by the forskolin effect) and were obtained in the presence of 0.5 μM forskolin except when the effect of CRF, individually or in combination with other reagents, was assayed. In panel C data are represented in percentage over basal (100% represented by a dotted line). Values are the mean ± SEM of 8 different experiments. In all cases, one way ANOVA followed by Bonferroni multiple comparison *post hoc* test showed a significant effect over basal (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

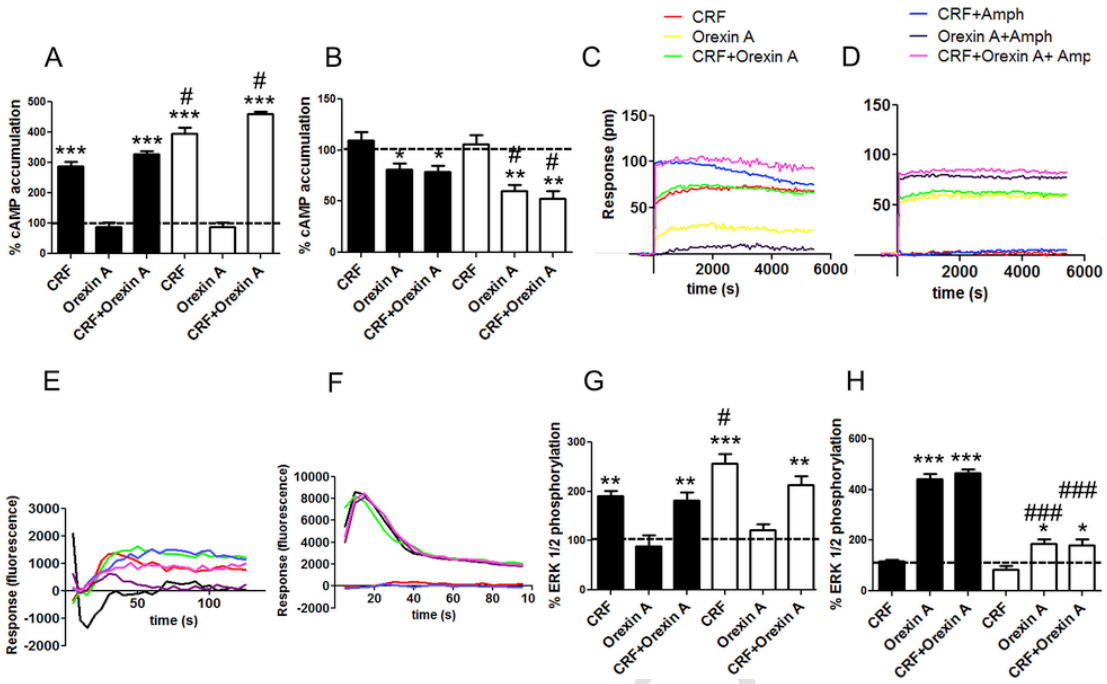

Fig. 3. Amphetamine potentiates both, orexin- and CRF-induced signaling. HEK-293T cells transfected with 0.75 μg cDNA for CRF_2 receptor (A, C, E, G) or with 1 μg cDNA for OX_1 receptor (B, D, F, H) were pretreated with 2 μM amphetamine (white bars) or vehicle (black bars) for 30 min prior to CRF (100 nM), orexin A (100 nM) or combined treatments. cAMP levels (A–B), calcium release (C–D), DMR (E–F) and ERK1/2 phosphorylation (G–H) data were collected. Data are given in percentage respect to basal (A) or respect to cAMP levels obtained in the presence of 0.5 μM forskolin (B); in A and B 100% a dotted line indicates the 100% value. Values are the mean ± SEM of 7 different experiments. In all cases, one way ANOVA followed by Bonferroni multiple comparison *post hoc* test showed a significant effect over basal (or over forskolin in the case of cAMP assays using orexin A)(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), or amphetamine treatment versus vehicle (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$).

to σ_1R. Accordingly, we wondered whether the amphetamine modulation of CRF_2R action could be mediated by σ_1R. First, HEK-293T cells were transfected with a constant amount of cDNA (0.3 μg) for σ_1R-RLuc and increasing amounts of cDNA for CRF_2R-YFP (0.1–1.5 μg). A positive and saturable BRET curve, indicative of dimer formation, was obtained (Fig. 4A). We subsequently addressed the potential of σ_1R to mediate the effect of amphetamine on CRF_2R by silencing σ_1R expression using a siRNA approach. Importantly, we used HEK-293T cells expressing CRF_2R with (Fig. 4D) or without the siRNA (Fig. 4C) to determine cAMP levels in cells pretreated for 30 min with vehicle (black bars) or with 2 μM amphetamine (white bars). The results showed that cells with silenced σ_1R, did not exhibit the amphetamine-modulating effect. It is assumed that amphetamine potentiation of CRF-induced cAMP signaling was due to binding of the drug to σ_1R and to the physical interaction between CRF_2 and σ_1 receptors (Fig. 4D).

2.5. Amphetamine effects over OX_1R depend on σ_2R expression

It is known that OX_1R are not able to physically interact with σ_1R (Navarro et al., 2015). Thus, it is difficult to understand how amphetamine could modulate Orexin A effects. It has been reported (Cobos et al., 2008; Nguyen et al., 2005) that different drugs of abuse may bind to σ_2R. To know whether amphetamine effects on OX_1R function could be due to the formation of a σ_2R-OX_1R complex, HEK-293T cells expressing a constant amount of OX_1R-RLuc (0.35 μg) and increasing amounts of σ_2R-YFP (0.05–0.4 μg) were assayed for BRET. A specific interaction between σ_2R and OX_1R receptors was deduced from the saturable BRET curve ($BRET_{max}$ 21 ± 4, $BRET_{50}$ 43 ± 14) (Fig. 4B). Functional studies were then undertaken in HEK-293T cells expressing OX_1R and endogenous σ_2R (Fig. 4C) or in cells in which the expression of σ_2R was silenced by a specific siRNA (Fig. 4E). Amphetamine (2 μM) pretreatment only modulated the action of Orexin A action on forskolin-induced cAMP levels when σ_2R was expressed. In summary, amphetamine potentiation of receptor-G_i coupling seemed due to the physical interaction between OX_1R and σ_2R.

2.6. Amphetamine potentiates the negative cross-talk displayed by the OX_1R-CRF_2R heteromer

We next moved to test the effect of the drug of abuse on the heteromer-mediated signaling. Immunocytochemistry assays were performed in a heterologous expression system to investigate whether amphetamine could affect the expression of OX_1R-CRF_2R heteromers. OX_1R was expressed at the plasma membrane level with no major changes in cells pretreated with amphetamine, CRF_2R, which showed a mainly intracellular expression in the absence of amphetamine, increased surface expression in cells treated with the drug. Moreover, colocalization between CRF_2R and OX_1R at the plasma membrane level increased (Fig. 5A). To analyze the amphetamine effect over CRF_2R-OX_1R heteromeric complexes, a BRET assay was performed in cells expressing a constant amount of OX_1R-RLuc and increasing amounts of CRF_2R-YFP. Similar results were obtained in cells untreated (black line) or treated with 2 μM amphetamine (red line) ($BRET_{max}$ 56 ± 2, $BRET_{50}$ 9 ± 1 and $BRET_{max}$ 62 ± 4, $BRET_{50}$ 10 ± 2, respectively) (Fig. 5B). These results indicate that amphetamine does not significantly alter the physical interaction between CRF_2R and OX_1R in the heteromeric complex. Finally, to assess any effect of amphetamine over the CRF_2R-OX_1R complex, cAMP, ERK1/2 phosphorylation, calcium mobilization and label-free assays were performed in cotransfected cells pretreated with receptor antagonists (1 μM K41498 or 1 μM SB334867) and with 2 μM amphetamine or vehicle. When receptors were activated using CRF (100 nM) or Orexin A (100 nM), amphetamine potentiated both CRF- and Orexin A-receptor-mediated. Remarkably, upon receptor coactivation, the negative cross-talk observed in the absence of amphetamine, was not only maintained in the presence of the drug but it was potentiated. Moreover, the cross-antagonism phenomenon described in Fig. 2

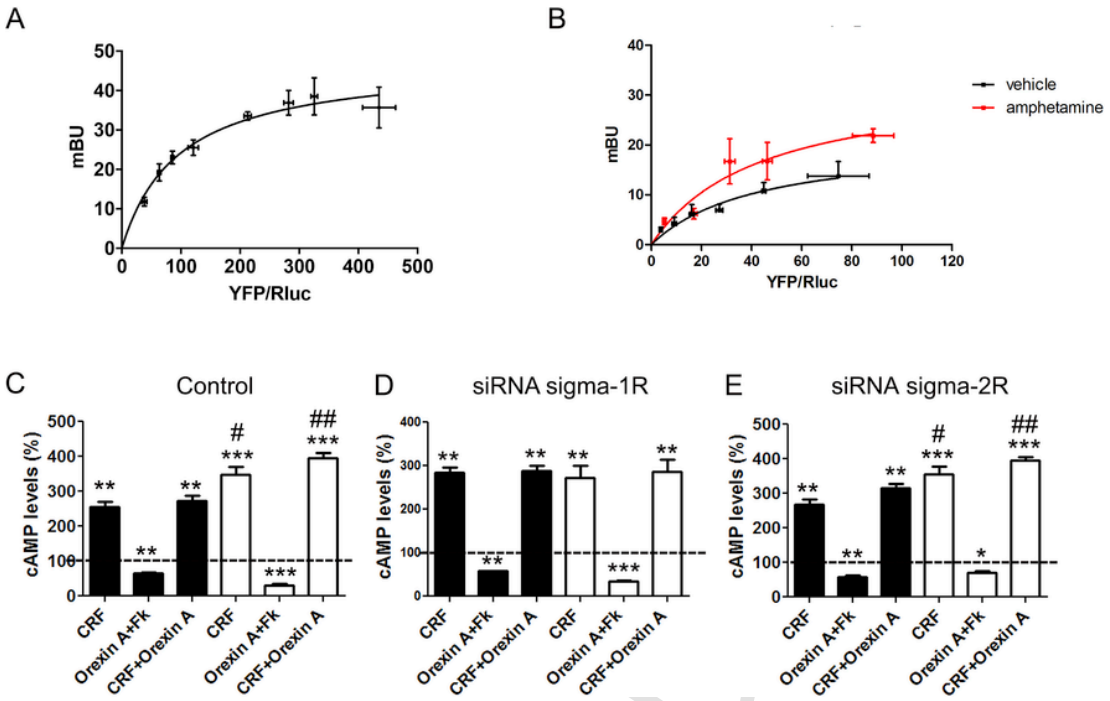

Fig. 4. CRF_2 receptors interact with σ_1 (sigma-1R) and OX_1 receptors interact with σ_2 receptors (sigma-2R). Panel A: HEK-293T cells were transfected with a constant amount of cDNA (0.3 μg) for σ_1R-RLuc and with increasing amounts of cDNA for CRF_2R-YFP (0.1–1.5 μg). Values are the mean ± SEM of 6 different experiments. Panel B: HEK-293T cells were transfected with a constant amount of cDNA (0.35 μg) for OX_1R-RLuc and with increasing amounts of cDNA for σ_2R-YFP (0.05–0.4 μg). Values are the mean ± SEM of 7 different experiments. Panels C–E: HEK-293T cells transfected with 0.5 μg cDNA for CRF_2 receptor and with 0.6 μg cDNA for OX_1 receptor in the absence (C) or presence of siRNA (RNAi) for σ_1R (D) or for σ_2R (E) were pretreated with 2 μM amphetamine (white bars) or vehicle (black bars) for 30 min prior to receptor activation using CRF (100 nM), orexin A (100 nM) or both. In panels C–E data are represented in percentage and were obtained in the presence of 0.5 μM forskolin except when the effect of CRF, individually or in combination with other reagents, was assayed (100% -dotted line-is the basal level or the level obtained by 0.5 μM forskolin treatment). Values are the mean ± SEM of 8 different experiments. In all cases, one way ANOVA followed by Bonferroni multiple comparison *post hoc* test showed a significant effect over basal (*$p < 0.05$**$p < 0.01$***$p < 0.001$, or amphetamine treatment versus vehicle (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$).

also appeared when amphetamine was added to HEK-293T cells coexpressing CRF_2R and OX_1R. Altogether, it can be concluded that amphetamine addition exacerbates CRF_2R-OX_1R heteromer-mediated signaling.

2.7. Microdialysis experiments showed that amphetamine potentiates Orexin A induced dopamine and glutamate release and in vivo cross-antagonism

The ability of amphetamine to modulate the OX_1R function was addressed in a more physiological setup, namely in microdialysis experiments performed in living animals. Data from these assays provide surrogate measures for dopamine and glutamate release and/or interstitial concentration in the VTA. Fig. 6A shows the typical placement of the microdialysis probe in the VTA, at 5.2 mm posterior to bregma. As it can be seen in Fig. 6B, in amphetamine-treated rats (white), infusion of Orexin A (10 μM) for 20 min to VTA produced an increase in extracellular dopamine (top) and glutamate release (bottom) compared to saline-control rats (black). These data agree with that obtained in HEK-293T cells indicating that amphetamine treatment potentiates OX_1R function. Remarkably, the increase of both extracellular dopamine (top) and glutamate levels (bottom) was counteracted by a previous VTA infusion of the CRF_2R selective antagonist K41498 (1 μM) (Fig. 6C), demonstrating the negative cross-antagonism previously described in HEK-293T cells. These results are probably the first to report a cross-antagonism in *in vivo* conditions.

3. Discussion

The results here presented provide evidence of a novel interaction involving two GPCRs, OX_1R and CRF_2R, in both heterologous expression systems and natural sources. Two allosterically-mediated specific properties of this heteroreceptor complex were i) a blockade by CRF of OX_1R signaling and ii) a cross-antagonism, which was instrumental to detect the heteromers even in living animals. A review on the uniqueness of GPCR heteromers as example of the ability of antagonists of one protomer to antagonize the signaling of another protomer in an heteroreceptor complex is provided elsewhere (Franco et al., 2016). The structure of complexes formed by GPCRs and coupled G proteins is instrumental for heteromer-function (Cordomí et al., 2015). The negative modulation of the effect of Orexin A by CRF suggests that some of the effects of Orexin A on the HPA (Spinazzi et al., 2006; Steiner et al., 2013) occur at the level of receptors forming heteromers in brain cells. Our results also constitute an underlying framework to address therapeutic approaches to combat stress-related psychiatric disorders in which the hypocretin/orexin system has a relevant role (reviewed in (James et al., 2017). It should be noted that the expression of the orexin receptor has been characterized in cells of the HPA axis (Czerwinska et al., 2017). We also demonstrate that amphetamine modulates both OX_1R and CRF_2R-mediated signaling and that the effect of the drug was mediated by sigma receptors forming heteromers with OX_1R and CRF_2R. The overall mechanism constitutes an example of cytocrin signaling that may underlie both short-term and long-term transcription-mediated events (Navarro et al., 2017).

Two are the sigma receptors identified to date: the non-opioid receptor, σ_1R, and σ_2R, whose identity with the Progesterone Receptor

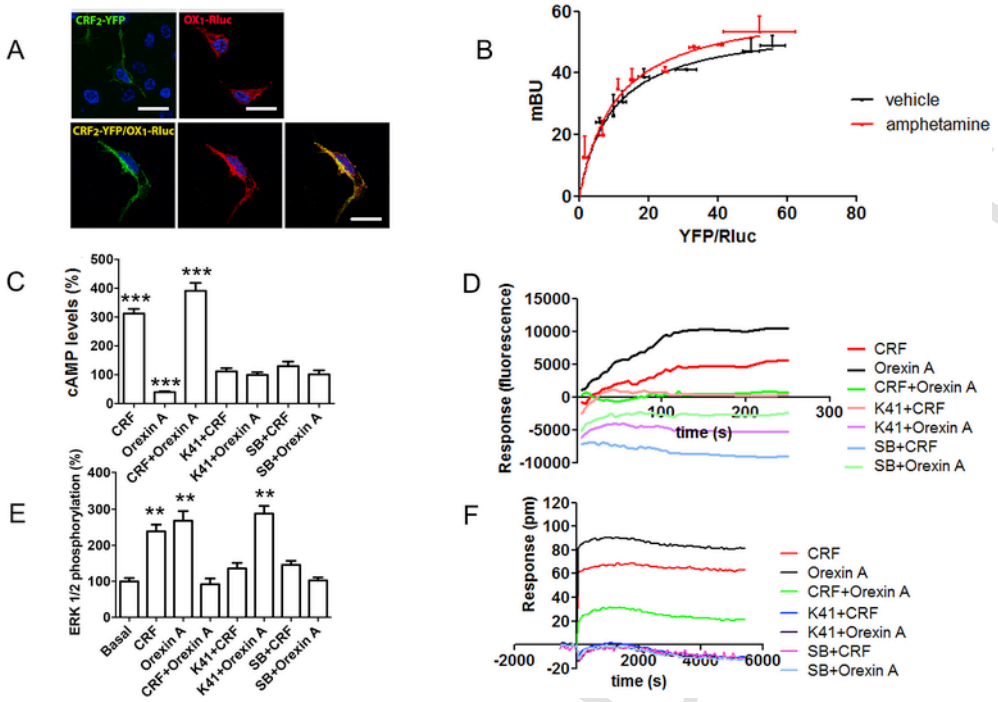

Fig. 5. Amphetamine effect over CRF_2-OX_1 heteroreceptor complexes. Panel A: HEK-293T cells transfected with 0.75 μg cDNA for CRF_2-YFP, 0.4 μg cDNA for OX_1-RLuc or both were pretreated with 2 μM amphetamine for 30 min. Receptors were identified by YFP fluorescence (green) or a monoclonal anti-RLuc (1/100) primary antibody and a cyanine-3-conjugated (1/200) secondary antibody (red). Colocalization of the two receptors is shown in yellow. Nuclei were stained with Hoechst (1/100, blue). Relative fluorescence intensity was 47 for CRF_2-YFP and 58 for OX_1-Rluc (SD < 15%) (see legend of Fig. 1 for fluorescence intensity comparison). Scale bar: 20 μm. Panel B: BRET was performed in HEK-293T cells transfected with a constant amount of cDNA (0.4 μg) for OX_1R-RLuc and increasing amounts of cDNA (0.2–1.5 μg) for CRF_2R-YFP and treated with 2 μM amphetamine (red line) or vehicle (black line) for 30 min prior to data recording. Values are the mean ± SEM of 7 different experiments. Panels C-F: HEK-293T cells transfected with 0.5 μg cDNA for CRF_2 receptor and 0.6 μg cDNA for OX_1 receptor were pretreated with 2 μM amphetamine (30 min), further treated with the CRF_2 receptor antagonist K41498 (1 μM) or the OX_1 receptor antagonist SB334687 (1 μM), and finally with CRF (100 nM), Orexin A (100 nM) or both. cAMP levels (C), calcium release (D), ERK1/2 phosphorylation (E) and DMR (F) data were collected. In panels C and E data are represented in percentage over basal. In panel C data were obtained in the presence of 0.5 μM forskolin except when the effect of CRF, individually or in combination with other reagents, was assayed. Values are the mean ± SEM of 8 different experiments. In all cases, one way ANOVA followed by Bonferroni multiple comparison *post hoc* test showed a significant effect over basal (*p < 0.05**p < 0.01***p < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Membrane Component 1 (PGRMC-1) protein is in doubt (Chu et al., 2015). Despite endogenous ligands have not been yet identified, drugs of abuse may interact with these two receptors at "physiologically" relevant concentrations. Recent studies have demonstrated that cocaine binding to σ_1R regulates GPCR signaling, likely, by means of direct interaction between the σ_1R and, among other, dopamine D_1 (Navarro et al., 2010) (Moreno et al., 2014) and D_2 receptors (Navarro et al., 2013) and corticotropin-releasing factor CRF_1 receptor (Navarro et al., 2015). Although its role as true receptor is not demonstrated, synthetic agonists and antagonists are available for σ_1R. PRE-084 is considered a selective agonist due to its ability to dose-dependently dissociate σ_1R from a binding immunoglobulin protein/78 kDa glucose-regulated protein (BiP/GPR-78) (Hayashi and Su, 2007). Once the agonist binds to σ_1R, the receptor translocates to the plasma membrane and modulates cell responses via protein-protein-interaction events related to ion handling (Su et al., 2016; Wu and Bowen, 2008). Of special interest here is the regulation of GPCR functionality. σ_1R not only binds cocaine but methamphetamine and, therefore, drugs of abuse use σ_1R to mediate some of their effects (Lever et al., 2016; Nguyen et al., 2005; Shull, 2002; Skuza, 1999). Drugs impeding the interaction of cocaine with σ_1R are proposed to reduce drug-seeking behavior (Matsumoto et al., 2001).

There is evidence of σ_2R involvement in amphetamine effect on dopamine transport (Izenwasser et al., 1998; Weatherspoon and Werling, 1999). In addition, treatment with σ_2R antagonists counteracts cocaine-induced locomotor stimulation in mice (Guo and Zhen, 2015; Lever et al., 2014). Our results now point to a relevant role of σ_2R on mediating effect of drugs of abuse and by mechanisms also involving interaction with GPCRs and modulation of their functionality. Remarkably, we here report that the effect of amphetamine on CRF_2R was mediated by σ_1R, whereas the effect of the drug on OX_1R was, instead, mediated by σ_2R. Although direct studies with amphetamine have not been reported, it is known that methamphetamine may bind to both sigma receptors although with more affinity to the σ_1R (Nguyen et al., 2005).

The results of microdialysis are relevant as they demonstrate that Orexin A leads in the VTA to increases in interstitial concentration of both dopamine and glutamate. The finding fits with hypocretin-mediated increased glutamatergic neurotransmission in this area (Borgland et al., 2008), with regulation of dopamine neuron activity driven by prefrontal cortex activation (Moorman and Aston-Jones, 2010), with fast scan cyclic voltammetry-based data on regulation of dopamine concentration in the nucleus accumbens shell (Patyal et al., 2012), and with regulation of synaptic plasticity elicited by morphine (Baimel and Borgland, 2015). Interestingly, CRF_2 receptor is expressed in VTA terminals of neurons projecting from the hypothalamus (Slater et al., 2016). The effect of local administration of amphetamine on increasing VTA dopamine concentration was already reported in the nineties (Byrnes and Wallace, 1997; Pan et al., 1996). Later on, differential effects due to different administration regimes of amphetamine (and of cocaine) administration was reported (Zhang et al., 2001). In addition a review on the evidence of glutamatergic and dopaminergic neurotransmission involvement in the behavioral actions exerted by amphetamine was provided (Vanderschuren and Kalivas, 2000). The mediation of $GABA_B$ receptors on the alteration of glutamate and dopamine efflux after amphetamine administration was also suggested (Giorgetti

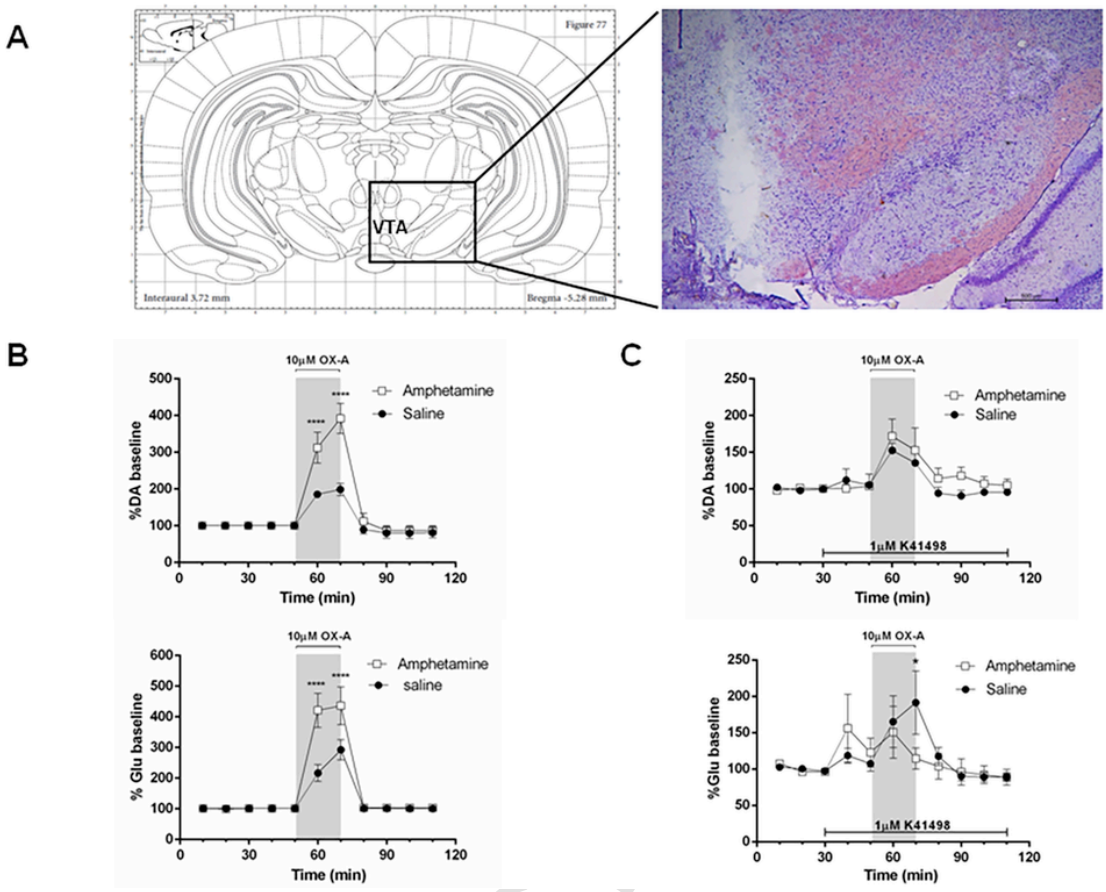

Fig. 6. Amphetamine potentiates orexin-induced extracellular glutamate and dopamine release in the VTA. Panel A: scheme extracted from the rat brain atlas (Paxinos and Watson, 1986) and showing the placement of the microdialysis probe, at 5.2 mm posterior to bregma, 0.7 lateral and 8.7 mm ventral. Panel B: Infusion in VTA of orexin A (10 μM) for 20 min (vertical grey bars) was followed by determination of dopamine (DA, top) and Glutamate (Glut, bottom) levels. Data from saline infusion are in black and data from amphetamine-treated rats are in white. Panel C: effect of VTA infusion of Orexin A (10 μM) for 20 min (vertical grey bars) after 20 min treatment with the CRF_2R antagonist K41489 (1 μM, horizontal black line) on extracellular dopamine (DA, top) and glutamate (Glu, bottom) release. Data from control rats are in black and data from amphetamine-treated rats are in white. In panels B and C data are represented in percentage over basal. Values are the mean ± SEM of 5 different experiments. In all cases, Two-way ANOVA followed by Bonferroni multiple comparison *post hoc* test showed a significant effect over basal (*$p < 0.05$; ****$< p < 0.0001$).

et al., 2002). Interestingly, evidence points that metabotropic but not ionotropic receptors in the nucleus accumbens, are involved in the amphetamine-mediated increase in dopamine efflux/levels (Darracq et al., 2001). It is however intriguing that amphetamine may block "*inhibitory glutamate transmission in dopamine neurons*" (Paladini et al., 2001).

Finally, our results suggest that σ_2R may, at least partially, mediate the hunger-suppressive action of amphetamine by interacting with orexigenic receptors in CRF_2R-OX_1R heteromer contexts. Despite extensive evidence supporting the formation of GPCR oligomers in heterologous systems, the lack of appropriate methodology makes controversial their existence in their native environment. A specific pharmacological property for the heterodimeric receptor complex is critical for identify such signaling complexes in native tissue. In the present study, we observed that Orexin A caused an increase of extracellular dopamine and glutamate levels in the VTA that was counteracted by a previous VTA infusion of a CRF2R selective antagonist. These microdialysis experiments demonstrate the negative cross-antagonism previously described *in vitro* and confirm that this dimeric entity is functionally relevant *in vivo*. To our knowledge, this is the first report showing *in vivo* GPCR heteromer identification by cross-antagonism. Thus, the present study conveys a novel approach to add to those that allow identification of GPCR heteromers in natural sources (Franco et al., 2016).

4. Materials and methods

4.1. Reagents

Orexin A, CRF, SB334867 and K41498 were purchased from Tocris Bioscience (Bristol, UK). DL-Amphetamine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). For the microdialysis experiments, amphetamine sulfate was donated by Laboratorio Chile S.A (Nuñoa, Santiago, Chile).

4.2. Cell lines

HEK-293T cells were grown in DMEM (Gibco, Thermo Fisher Scientific, Halethorpe, MD) supplemented with 2 mM L-glutamine, 100 μg/ml sodium pyruvate, 100 units/mL penicillin/streptomycin, minimum Eagle's medium non-essential amino acid solution (1/100) and 5% (v/v) heat-inactivated Fetal Bovine Serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK).

4.3. Expression vectors and fusion proteins

Human cDNAs for CRF_2 receptor, OX_1 receptor, σ_1 receptor, σ_2 receptor and GHS-R1a cloned into pcDNA3.1, were amplified without their stop codons using sense and antisense primers harboring the fol-

lowing: EcoRI and KpnI sites to clone OX_1 receptor and σ_2 receptor in the pcDNA3.1RLuc vector (pRLuc-N1, PerkinElmer Life Sciences, Wellesley, MA) and to clone CRF_2 receptor and GHS-R1a in the pEYFP–N1 vector (enhanced yellow variant of GFP; Clontech, Heidelberg, Germany); HindIII and BamHI sites to clone σ_1R in pEYFP–N1 vector; EcoRI and BamHI sites to clone A_{2A} receptor in pEYFP–N1 vector. Amplified fragments were subcloned to be in-frame with restriction sites of pRLuc-N1 or pEYFP-N1 vectors to provide plasmids that express proteins fused to RLuc or YFP on the C-terminal end (OX_1-RLuc, CRF_2-YFP, GHS-R1a-YFP, σ_1R-RLuc and σ_2R-RLuc).

4.4. Cell transfection

HEK-293T cells growing in 6-well dishes were transiently transfected with the corresponding protein cDNA by the PEI (Sigma-Aldrich, St. Louis, MO) method. Cells were incubated with the corresponding cDNA together with PEI (5.47 mM in nitrogen residues) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. Cells were maintained at 37 °C in a humid atmosphere of 5% CO_2.

4.5. Resonance energy transfer-based assays

For BRET assays (Hinz et al., 2018), HEK-293T cells were transiently co-transfected with a constant amount of cDNA encoding for receptor-RLuc and with increasing amounts of cDNA corresponding to receptor-YFP. 24 h after transfection, cells were adjusted to 20 μg of protein using a Bradford assay kit (Bio-Rad, Munich, Germany) and bovine serum albumin for standardization. To quantify fluorescence proteins, cells were distributed in 96-well black microplates with a transparent bottom (Corning 3600, Corning, NY), and the fluorescence was read in a Fluostar Optima fluorimeter (BMG Labtech, Offenburg, Germany) equipped with a high-energy xenon flash lamp using a 10 nm bandwidth excitation filter at 485 nm for receptor-YFP reading. Receptor fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing receptor-RLuc alone. For BRET measurements, cells were distributed in 96-well white microplates with white bottoms (Corning) and 5 μM of coelenterazine H (Molecular Probes, Eugene, OR) was added. One minute after adding coelenterazine H, BRET was determined using a Mithras LB 940 reader (Berthold Technologies, Bad Wildbad, Germany), 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 expression, luminescence readings were performed 10 min after 5 μM coelenterazine H addition using a Mithras LB 940. 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. GraphPad Prism software (San Diego, CA, USA) was used to fit data. BRET is expressed as mili BRET units, mBU (net BRET x 1000).

4.6. Immunocytochemistry

Transiently transfected HEK-293T cells were incubated 30 min with vehicle or 2 μM amphetamine and immediately, cells were fixed in 4% paraformaldehyde for 15 min and washed twice with PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. After permeabilization with buffer A containing 0.2% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin. After 1 h at room temperature, cells expressing receptor-RLuc were labeled with a primary mouse monoclonal anti-RLuc antibody (1/100, EMD Millipore, Darmstadt, Germany) for 1 h, washed, and stained with a secondary antibody for Cy3 donkey anti-mouse (1/100, Jackson ImmunoResearch Laboratories, Baltimore, MD). Receptors fused to YFP were detected by their fluorescent properties. Cell nuclei were stained with Hoechst (Sigma-Aldrich). Samples were washed, mounted with 30% Mowiol (Calbiochem) and observed under a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany). Using the Fiji package (pacific. mpi-cbg.de), fluorescence intensity was analyzed in the different channels in 5 areas of 10 different cells.

4.7. Dynamic mass redistribution assays (DMR)

Cell mass redistribution induced upon receptor activation were detected by illuminating the underside of a biosensor with polychromatic light and measuring the changes in the wavelength of the reflected monochromatic light that is a sensitive function of the index of refraction. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. HEK-293T cells were seeded in 384-well sensor microplates to obtain 70–80% confluent monolayers constituted by approximately 10,000 cells per well. Previous to the assay, cells were washed twice with assay buffer (HBSS with 20 mM HEPES, pH 7.15) and incubated 2 h with assay-buffer (24 °C, 30 μl/well). Hereafter, the sensor plate was scanned and a baseline optical signature was recorded for 10 min before adding 10 μl of amphetamine or the specific antagonists 30 min prior to agonist stimulation; all test compounds were dissolved in assay buffer. The cell signaling signature was determined using an EnSpire® Multimode Plate Reader (PerkinElmer) by a label-free technology. Then, DMR responses were monitored for at least 4000 s. Results were analyzed using EnSpire Workstation Software v 4.10.

4.8. Intracellular calcium release

Cells were co-transfected with the cDNA for the indicated receptors and 0.75 μg of GCaMP6 calcium sensor (Chen et al., 2013) using the PEI method. 24 h after transfection, 150,000 cells/well in 96-well black, clear-bottom microtiter plates were incubated with Mg^{+2}-free Locke's buffer (pH 7.4) (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose and 5 mM HEPES) supplemented with 10 μM glycine, and receptor ligands were added as indicated (antagonists were added 10 min bafore agonist stimulation). The fluorescence emission intensity of GCaMP6 was recorded at 515 nm upon excitation at 488 nm on the EnSpire® multimode plate reader for 335 s every 15 s and 100 flashes/well.

4.9. cAMP Accumulation

Homogeneous time-resolved fluorescence energy transfer assays were performed using the Lance Ultra cAMP kit (PerkinElmer Life Sciences) (Navarro et al., 2018, 2016). The optimal cell density was first established for an appropriate fluorescent signal by measuring the time-resolved FRET signal as a function of 0.5 μM forskolin concentration using different cell densities. Forskolin dose-response curves were related to the cAMP standard curve to establish which cell density provides a response that covers most of the dynamic range of the cAMP standard curve. Cells growing in medium containing 50 μM zardeverine were pretreated with vehicle or 2 μM amphetamine for 30 min at 25 °C. Then, 3000 HEK-293T cells/well in 384-well microplates were treated with the antagonists or the corresponding vehicle for 15 min and stimulated with agonists for 15 min before adding 0.5 μM forskolin or vehicle and incubating for an additional 15-min period. Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with a homogeneous time-resolved fluorescence energy transfer optical module (BMG Labtech).

4.10. ERK phosphorylation assays

To determine ERK1/2 phosphorylation, 40,000 cells/well were placed in transparent Deltalab 96-well microplates and kept at the incubator for 24 h. Then, cells were transfected using the PEI method and incubated for a 48 h period. 2–4 h before the experiment, the medium was substituted by serum-starved DMEM medium. Then, cells were pre-treated at 25 °C for 10 min with vehicle or antagonists in serum-starved DMEM medium and treated for 7 min with agonists. Cells were then washed twice with cold PBS before addition of lysis buffer (20 min treatment in agitation). 10 μL of each supernatant were placed in white ProxiPlate 384-well microplates and ERK 1/2 phosphorylation was determined using AlphaScreen®SureFire® kit (Perkin Elmer) following the instructions of the supplier and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA).

4.11. Animals

Male Sprague-Dawley rats weighing 200–230 g were selected for the experiments. They were kept in a controlled environment with a 12-h light-dark cycle and at 21 °C room temperature. Food and water were provided *ad libitum*. All experimental procedures were approved by the Ethics Committee of Faculty of Biological Sciences of "Pontificia Universidad Católica de Chile" and follow the international guidelines (NIH Guide for the Care and Use of Laboratory Animals).

4.12. Experimental procedure

Animals keep housing and handling in colony for 3 days once of arrived and then were divided randomly into two groups that received injection of either AMPH (1.5 mg/kg, i.p) or an equivalent volume of saline solution once per day. The procedure was carried out at 10:00–11:00 a.m., once a day for a five consecutive days. After 5 days of abstinence, microdialysis experiment was performed.

4.13. Microdialysis and analysis of samples

The animals were deeply anesthetized with chloral hydrate (400 mg/kg, i.p). Body temperature of the animals was maintained at 37 °C with an electrical blanket controlled by a thermostat. A quarter of the initial dose of chloral hydrate was given every hour to maintain the animal anesthetized during the course of the experiments. Concentric brain microdialysis probes, 2 mm in length (CMA 12, CMA Microdialysis AB, Solna, Sweden) were implanted in the VTA. The coordinates used, according to the atlas of Paxinos and Watson (1986), were: VTA: antero-posterior (AP): 5.2 mm, medial-lateral (ML) 0.7 mm and dorso-ventral (DV) −8.7 mm. Microdialysis probes were perfused with Krebs-Ringer´s phosphate buffer whit 0.02% BSA (KRP-BSA) at a rate of 2 μL/min using a Harvard infusion pump (Model 22; Dover, MA, USA). After a 90 min stabilization period, 10 min samples were collected in 4 μL of 0.2 M perchloric acid. K411498 (1 μM) and orexina A (10 μM) were added intra-VTA in the perfusion media as indicated in the respective figures. At the end of each experiment, animals were killed by decapitation and brains quickly removed and stored in formalin. Brain coronal sections of 30 μm were stained with Cresyl violet to verify probe location under microscope.

4.13.1. Analysis of dialysate samples

HPLC-amperometric quantification of dopamine was performed as described previously (Galleguillos et al., 2010). HPLC-fluorometric determination for amino acids was performed as described previously (Sotomayor-Zarate et al., 2010).

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HPLC-electrochemical determination of dopamine was performed as previously described (Sotomayor et al., 2005). The retention time for dopamine was 11.0 min and the detection limit was 0,1 fmol/μL. HPLC-fluorometric determination of glutamate was performed as previously described (Sotomayor-Zárate et al., 2010). The retention time for glutamate was 2.8 min and the detection was 5 fmol/μL.

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