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Anjani Chitrapu April 12th, 2011

Interactions Between Dopaminergic and Adrenergic Receptors

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Abstract

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Cocaine facilitates dopaminergic and noradrenergic signaling throughout the brain, which are critical for mediating different aspects of cocaine addiction. Both of these neurotransmitters are released in the prefrontal cortex (PFC), a brain region that has been linked to reinstatement of drug-seeking behavior, which models relapse in humans. In particular, the D1-dopamine receptor (D1DR) and α1-adrenergic receptor (α1AR) have been implicated in relapse-like behavior. Pharmacological and biochemical interactions between these receptors have been reported in the PFC, although the exact nature of these interactions is not clear. Additionally, the α1bAR subtype of AR has been linked to neurochemical and behavioral responses to cocaine. Previous research in our lab has shown that D1DR and α1bAR co-localize to dendrites in the PFC, suggesting that the interactions occur cell-autonomously. We hypothesized that D1DR and α1bAR physically interact by forming heterodimers. Using co-immunoprecipitation studies in transfected HEK293 cells, it was confirmed that D1DR and α1bAR are capable of forming stable complexes. We also found evidence for heterodimer formation between other AR and DR subtypes. Given the physical interaction between D1DR and α1bAR, we tested whether agonist-induced internalization of one receptor would alter trafficking of the other receptor. Using a surface luminescence assay, we found that treatment with the α1AR agonist phenylephrine induced ~ 30% internalization of the α1bAR when expressed alone or in combination with D1DR. By contrast, DR stimulation with dopamine increased surface expression of α1bAR only in cells co-expressing both receptors. These results suggest that dopaminergic and adrenergic receptors are capable of forming a complex, and that the trafficking of these receptors may be altered in the heterodimer form.

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INTRODUCTION

Cocaine is a psychostimulant drug with a very strong addictive potential. Over 36.8 million Americans over the age of twelve have used cocaine at least once in their lifetime (National Survey on Drug Use and Health, 2008). The societal implications of cocaine abuse are high, and in 2004, 46.8% of state prisoners indicated having used cocaine at some point in their lives (Bureau of Justice Statistics, 2004). Part of the addictive nature of cocaine and other psychostimulants is a result of the brain's reward system being over-activated upon use of the drug. This causes mood elevation, and renders the drug's effects pleasurable, increasing the desire of its continued use and the likelihood of addiction (Wise, 1984).

Dopamine and norepinephrine function in cocaine addiction

The brain's reward system comprises a network of dopaminergic projections from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAc), prefrontal cortex (PFC), and the ventral pallidum (Wise, 1984). Cocaine blocks presynaptic monoaminergic neurotransmitter transporters within the reward system and elsewhere, impeding reuptake of dopamine (DA), as well as norepinephrine (NE) and serotonin (5HT), at the axon terminals from which they are released (Ritz et al, 1987). This transporter blockage causes the neurotransmitter to persist in the synaptic cleft, resulting in prolonged activation of DA, NE, and 5HT receptors on target cells. One consequence of the neurochemical actions of cocaine is enhancement of DA transmission in the NAc, which has been implicated in the rewarding properties of cocaine. The intravenous self-administration (IVSA) paradigm is widely used to assess the reinforcing effects of addictive drugs, including cocaine. In this paradigm, an animal performs an operant behavior such as lever-pressing to receive an intravenous injection of a drug. It has been shown that removing dopaminergic input to the NAc in rats, via 6-

hydroxydopamine (6-OHDA) lesions, decreases both the stimulatory locomotor effects of cocaine (Kelly et al, 1976), as well as IVSA of the drug (Pettit et al, 1984). Therefore, increased dopaminergic signaling in the NAc is critical for initial drug reward.

Although cocaine blocks NE transporters in addition to DA transporters, the resulting elevation of NE does not appear to contribute to the primary reinforcing effects of cocaine (Roberts et al, 1977). Instead, NE mediates the reinstatement phase of IVSA, which models relapse in humans. In the IVSA paradigm, stable responding for cocaine can be extinguished by removing the cocaine supply and replacing it with saline. After the rat decreases its drug-seeking behavior due to the lack of reinforcing stimulus, the drug-seeking behavior can be reinstated upon exposure to a non-contingent drug injection, stress, or presentation of drug-associated cues. Decreasing NE release by activating inhibitory receptors on noradrenergic cells attenuates stress-induced reinstatement in rats (Erb et al, 2000), and inhibition of dopamine β-hydroxylase, an enzyme required for NE synthesis, diminishes cocaine-primed reinstatement of cocaine-seeking in rats (Schroeder et al, 2010). Thus, although NE is not critical for mediating cocaine's initial rewarding properties, NE neurotransmission is necessary for reinstatement of cocaine-seeking behavior, and has implications in providing a potential target for pharmaceutical therapies to prevent relapse in humans.

Prefrontal cortex function in reinstatement of cocaine-seeking behavior

The PFC is a brain region critical for regulating behaviors such as impulsivity, working memory, and control of aggression (Sala et al, 2010), and dysfunction of the PFC is associated with psychiatric disorders such as schizophrenia, post-traumatic stress disorder, and attention-deficit hyperactivity disorder (Goto et al, 2009; Arnsten, 2007). The PFC is also important in mediating relapse of cocaine addiction in humans.

For example, the PFC is hypoactive at baseline in drug addicts, but becomes overactive upon exposure to drug-related stimuli (Goldstein and Volkow, 2002). In rats, temporary inactivation of the PFC by facilitation of inhibitory GABA-ergic transmission attenuates reinstatement of drug-seeking behavior elicited by drug prime, stress, or drug-associated cues (McFarland and Kalivas, 2001; McLaughlin and See, 2003; Capriles et al, 2003).

Both the dopaminergic and noradrenergic systems, which are shown to be critical for regulating different aspects of cocaine addiction, have projections terminating in the PFC (reviewed by Wienshenker and Schroeder, 2007). In the PFC, interactions between these systems of neurotransmission are involved in the neurochemical and behavioral effects of addictive drugs. For example, depletion of NE or pharmacological blockade of α1-adrenergic receptors (α1AR) in the PFC attenuates psychostimulant-induced locomotor activity and DA release in the NAc (reviewed by Schroeder and Weinshenker, 2007). The exact mechanisms by which dopaminergic and noradrenergic transmission influence each other in the PFC are being investigated. For example, activation of α1ARs regulates D1-dopaminergic receptor (D1DR) function in cultured PFC neurons by accelerating resensitization of D1DRs following desensitization induced by chronic DA exposure (Trovero et al., 2004). In addition, it has been shown that antagonizing α1ARs causes decreased signal transduction activity of D1DRs in the PFC (Gioanni et al, 1998). These results indicate that activity in the PFC may be modulated by direct interactions between dopaminergic and adrenergic receptors. The underlying anatomical and biochemical mechanisms are, however, not yet known. By understanding these interactions, more light can be shed on the involvement of NE and DA in the PFC in triggering relapse in cocaine addicts.

Dopaminergic and adrenergic receptors

Dopaminergic receptors (DR) and adrenergic receptors (AR) are members of the G-protein coupled receptor (GPCR) family, which are receptor proteins with seven transmembrane domains that activate downstream cascades upon ligand-binding, starting with activation of an associated G-protein (reviewed by Böhme and Beck-Sickinger, 2009). There are many different subtypes of both DRs and ARs. DRs fall into one of two families: D1-like or D2-like. The former includes D1 and D5 receptors (G_s-coupled), and the latter includes D2, D3, and D4 receptors (G_i-coupled; Missale et al, 1998). The AR types include α1 (G_q-coupled), α2 (G_i-coupled), β1 (G_s-coupled), β2 (G_s- and G_i-coupled), and β3 (G_s-coupled). Each receptor type comprises several subtypes as well. For example, the subtypes of α1AR are α1a, α1b, and α1d (Bylund et al, 1994).

Of these numerous DR and AR subtypes, D1DRs and α1ARs have been most extensively studied regarding interactions in the PFC. For example, blocking α1ARs in the medial PFC of rats via local infusion of an α1AR antagonist reduces psychostimulant-induced locomotion, a behavior that is primarily mediated by DA (Blanc et al, 1994). These results indicate that an interaction between the dopaminergic and noradrenergic systems in the PFC involves α1ARs. While there are three subtypes of α1ARs, α1bAR function has been specifically linked to the increased DA levels and behavioral responses following psychostimulant administration in mice. For example, α1bAR-knockout (KO) mice display attenuated neurochemical and behavioral responses to psychostimulants (Auclair et al, 2002). Likewise, antagonizing D1DRs in the PFC attenuates cocaine-seeking in rats, while blocking D2DRs showed non-specific effects on goal-directed behaviors (Sun and Rebec, 2005). These findings collectively indicate

a critical role for D1DRs and α1bARs in mediating PFC function and responses to drugs of abuse.

HYPOTHESIS

There is a body of evidence indicating that D1DRs and α1ARs in the PFC are critical for drug-seeking behaviors and that they mediate each other's functionality in this region. Double-labeling immunohistochemical data at the electron microscopic level, from our lab, indicates that α1ARs are found in ~70% of D1-expressing dendrites in rat PFC neurons (Mitrano et al, 2010). Given this high degree of colocalization, as well as the fact that GPCRs, including some subtypes of DRs and ARs, have been shown to form heterodimers (reviewed by Milligan, 2006), we hypothesized that a physical interaction between D1DRs and α1bARs could mediate, at least in part, the functional interactions seen between these receptors, both *in vitro* and *in vivo*. Because of previous studies implicating α1bARs in mediating DA-induced responses to psychostimulants (Auclair et al, 2002), as well as double-labeling immunohistochemical data showing evidence of a high degree of α1bAR localization in D1DR-expressing dendrites of rat PFC neurons (Mitrano et al, 2010), we predicted that D1DRs would specifically interact with α1bARs.

Secondly, it was hypothesized that co-expression of D1DRs and α1bARs would cause functional changes in receptor trafficking and/or signaling as a result of heterodimer formation. One aspect of functionality that can be altered by the presence of heterodimers is receptor co-internalization following ligand exposure (reviewed by Böhme and Beck-Sickinger, 2009). Immunohistochemical data from our lab shows that in rat PFC dendrites co-expressing α1bAR and D1DR, there is a higher ratio of intracellular:plasma membrane-bound α1bAR than in dendrites where α1bAR is

expressed alone, indicating that co-expression of α1bAR and D1DR alters surface expression of the receptors. Based on these data, we predicted that the presence of D1DR may alter α1bAR trafficking, and vice versa. For example, α1bAR may show internalization upon agonist stimulation of the DR, and in the presence of α1bAR, D1DR may internalize upon agonist stimulation of the AR.

RESEARCH AIMS

Aim 1. Determine whether D1DR and α1bAR can physically associate using co-immunoprecipitation studies in human embryonic kidney (HEK293) cells. In these experiments, HEK293 cells are transfected with hemagglutinin (HA) or FLAG epitope-tagged cDNA constructs encoding both receptors. One receptor (e.g. D1DR) is immunoprecipitated using a tag-specific antibody that is conjugated to agarose beads. The protein immunoprecipitate sample is then separated via electrophoresis, and a Western blot is performed. The blot is probed using an antibody specific to the receptor not directly immunoprecipitated by the antibody-conjugated agarose beads (e.g. α1bAR), and is visualized with an enhanced chemiluminescent substrate.

Aim 2. Assess specificity of D1DR-α1bAR binding by studying interactions between other subtypes of DRs and ARs. These experiments will involve the same transfection, co-immunoprecipitation, Western blot, and visualization methodology as above to study interactions of D1DR with α1aAR, D2DR with α1bAR, and D2DR with α1aAR.

Aim 3. Identify changes in agonist-induced receptor internalization when D1DR and α1bAR are co-expressed in HEK293 cells using a surface luminescence assay. This assay uses HEK293 cells that have been transfected with cDNA encoding D1DR and α1bAR, and are treated with either a DR or AR agonist. A horseradish peroxidase

(HRP)-conjugated antibody that binds only to surface receptors is applied, and emits a chemiluminescent signal, which can be read and quantified. These results will be compared between conditions in which D1DR and α1bAR are co-expressed, versus baseline conditions in which α1bAR or D1DR is expressed alone, to indicate changes in surface levels of expression. The results from agonist stimulation will be compared to a vehicle treatment, to allow for assessment of internalization.

METHODS

Transfection- In Dulbecco's Modification of Eagles Medium (DMEM) High Glucose with Glutamax (Invitrogen, Carlsbad, CA), confluent HEK293 cells are transfected, in 10 cm plates, with 2 µg of cDNA and 15 µL of Lipofectamine 2000 (Invitrogen), which is used to facilitate uptake of cDNA by the cells. In experiments using anti-FLAG agarose beads for immunoprecipitation, the following transfection conditions are followed: one cell plate is transfected with 2 µg of pcDNA that does not encode either receptor of interest. The second plate is transfected with 1 µg of pcDNA and 1 µg of HA-tagged DR-encoding cDNA. The third plate is transfected with 1 µg of FLAG-tagged AR-encoding cDNA, and 1 µg of HA-tagged DR-encoding cDNA.

When using anti-HA agarose beads for immunoprecipitation, the second group is transfected with 1 µg of pcDNA and 1 µg of FLAG-tagged AR-encoding cDNA. The cell cultures are incubated overnight at 37˚C.

Co-immunoprecipitation- After 24-48 hours, the transfected cells in each of the three conditions are collected and solubilized for 30-60 minutes in a harvest buffer made of 5% 1 M NaCl, 2% 250 mM EDTA, 1% 1M HEPES, 1% Triton-X, one Complete EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), and a small amount of benzamidine hydrochloride hydrate (Fischer Scientific, Fairlawn, NJ).

The samples then go through a high-speed centrifugation, which separates the membrane fraction into a pellet, while the soluble lysate remains as the supernatant. The membrane fraction and 50 µL of the soluble lysate are kept for analysis of membrane solubilization and transfection efficiency, respectively. The remaining soluble lysate is incubated with either anti-FLAG or anti-HA agarose beads for two hours (Table 1). Harvest buffer is used to wash the agarose beads of non-interacting proteins. The proteins that remain bound to the agarose beads are then eluted from the beads by boiling at 100˚C, and are separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Two gels are run using identical samples, and two separate nitrocellulose membranes are obtained, also with identical samples.

Western Blot- The nitrocellulose membranes are washed for 30 minutes in a blocking milk containing 5% 1M NaCl, 1% 1M HEPES, 0.1% Tween-20, and 10g dry milk for a 500mL solution. One membrane is incubated with an antibody specific to the AR, while the other membrane is incubated with an antibody specific to the DR for one hour each. Both antibodies are diluted in blocking milk. Primary antibody concentrations vary, and have been previously tested in the lab for optimal signal efficiency (Table 1). The nitrocellulose membranes are then washed 3 times with blocking milk for 5 minutes each. An HRP-conjugated secondary antibody, specific for the species in which the primary antibody was produced, is then applied for thirty minutes. Each of the secondary antibodies is diluted in blocking milk to a concentration of 1:4000. The membranes are washed 3 times with blocking milk for 7 minutes each, and are then coated in either a Supersignal West PICO chemiluminescent substrate or a Supersignal ELISA PICO chemiluminescent substrate (Thermo Scientific, Rockford, IL) visualization solution. The chemiluminescence is then used to expose autoradiographic film, which allows for

visualization of the samples. Receptor interactions will be confirmed if immunoprecipitation using agarose beads conjugated with an antibody specific for the tag on one receptor (e.g. HA tag on DR) results in visualization of the other receptor (e.g. AR).

Surface Luminescence Assay- HEK293 cells are transfected as described above. The following conditions are used when assessing AR internalization: One plate is transfected with 2 µg of pcDNA that does not encode either receptor of interest. The second plate is transfected with 1 µg of pcDNA and 1 µg of FLAG-tagged AR-encoding cDNA. The third plate is transfected with 1 µg of FLAG-tagged AR-encoding cDNA, and 1 µg of HA-tagged DR-encoding cDNA. 24 hours after transfection, cells are split onto 35 mm plates so that multiple agonist stimulations can be tested in each of the transfection conditions. One extra plate is split per transfection condition to be used in confirmation of transfection.

To conduct the assay, the cells must first be washed in PBS with Ca^{+2}. 1 mL of 50 µM agonist solution is then applied to the plates and allowed to incubate for 30 minutes. The AR agonist used is phenylephrine hydrochloride (Sigma-Aldrich, St. Louis, MO) and the DR agonist used is dopamine (Sigma-Aldrich). PBS with Ca^{+2} is used for vehicle treatment. The agonists are then removed and the plates are washed in PBS with Ca^{+2}. Next, 1 mL of 4% paraformaldehyde (PFA) is applied to the plates and allowed to incubate for 30 minutes to fix the cells. The cells are washed twice using PBS with Ca^{+2}, and are then incubated for another 30 minutes with 1 mL of 2% blocking milk. The blocking milk is then replaced with 1 mL of an anti-FLAG M2-HRP antibody dilution in blocking milk (1:1000) and allowed to incubate for one hour. The antibody solution is then removed and the plates are washed twice with 2% blocking milk. The cells are coated in PBS with Ca^{+2} and are prepared for reading in the luminometer.

Before each plate is read, the PBS with Ca^{+2} is removed, and replaced with a mixture of 800μL ELISA PICO solution, which is allowed to react with the HRP for 15 seconds, and is then removed. The plate is placed in the luminometer, and a reading is obtained in arbitrary units.

To confirm transfection, cells are collected from one plate per transfection in 150 μL 1X sample buffer and are sonicated in preparation for SDS-PAGE. 15 μL of each sample are separated via SDS-PAGE and transferred to a nitrocellulose membrane. A Western blot is conducted as described in the previous methodology, using antibodies against the receptors which were transfected into the cells.

RESULTS

Assessment of physical interactions between dopaminergic and adrenergic receptors

α1bAR & D1DR

To confirm expression of the transfected receptors in HEK293 cells, a sample of the soluble lysate was analyzed. When the Western blot of the soluble lysate samples from cells in each transfection group was probed with the α1bAR antibody, a robust 57 kDa band, corresponding to the predicted molecular weight of α1bAR (Table 2), was seen in the soluble lysate from cells transfected with α1bAR (Fig. 1A). Interestingly, fainter bands at 57 kDa were also seen in the soluble lysate from cells not transfected with α1bAR, which probably represents low levels of endogenous α1bAR expression. In these blots, bands can also be seen around 114 kDa, consistent with the predicted size of the α1bAR homodimer. When the blot was probed with the D1DR antibody, a 50 kDa band, corresponding to the predicted molecular weight of D1DR, was evident in all cells transfected with D1DR (Fig. 1B). For the immunoprecipitation experiments, we next

confirmed that the anti-FLAG agarose beads bind to FLAG-tagged α1bAR. When the Western blot of immunoprecipitate samples was probed with the α1bAR antibody, α1bAR was detected in the samples from cells co-expressing α1bAR and D1DR, but not in mock-transfected cells or those expressing D1DR alone (Fig. 1C). This indicates that only the epitope-tagged α1bAR, not any endogenously expressed α1bAR, is immunoprecipitated by the anti-FLAG agarose beads. When anti-FLAG agarose beads were used to immunoprecipitate α1bAR and the Western blot was probed with an antibody against D1DR, it was found that D1DR co-immunoprecipitates with α1bAR in cells expressing both receptors (Fig. 1D), indicating that α1bAR and D1DR interact physically and form a stable complex. Other bands in each of the blots likely represent various glycosylated forms of the receptors, homodimers, heterodimers, and degradation fragments of the receptors, all of which were detected by the antibodies.

These results were replicated by immunoprecipitating D1DR using anti-HA agarose beads and probing the Western blot of immunoprecipitate samples with the α1bAR antibody. When the soluble lysate samples were analyzed to confirm transfection, bands at 50 kDa were seen on the Western blot probed with a D1DR antibody in the cells transfected with D1DR (Fig. 2A). When the blot was probed with an α1bAR antibody, bands were seen at 57 kDa in the soluble lysate of cells transfected with α1bAR. Bands at 57 kDa were also seen in the mock-transfected cells, probably due to endogenous expression of α1bAR in HEK293 cells (Fig. 2B). When anti-HA agarose beads were used to immunoprecipitate D1DR, a band was seen at 50 kDa in the cells transfected with D1DR, but not in the cells expressing α1bAR alone or the mock-transfected cells, when the Western blot of immunoprecipitate samples was probed with a D1DR antibody (Fig. 2C). This confirms that D1DR binds to the anti-HA agarose beads. When anti-HA agarose beads were used to immunoprecipitate D1DR, α1bAR

co-immunoprecipitated with D1DR, and a band at 57 kDa can be seen on the Western blot of the immunoprecipitate samples from cells co-expressing the receptors (Fig. 2D). This result confirms that α1bAR and D1DR form a complex when anti-HA agarose beads are used to immunoprecipitate the receptors. Three replicates of each experiment were performed.

α1bAR & D2DR

Transfection of α1bAR and D2DR in HEK293 cells was confirmed by analysis of soluble lysate samples from cells in each transfection group. When an antibody against α1bAR was used to probe the Western blot of soluble lysate samples, a signal at 57 kDa was seen in the cells expressing α1bAR (Fig. 3A). Bands at 57 kDa were also seen in the cells not transfected with α1bAR, because of the likely endogenous expression of α1bAR in HEK293 cells. The blot was probed with an antibody against the HA-tag on D2DR, because D2DR antibodies are notoriously poor and do not work well in these experiments, and a band at 50 kDa was seen in cells transfected with D2DR (Fig. 3B). Using anti-FLAG agarose beads to immunoprecipitate α1bAR, the Western blot of immunoprecipitate samples, probed with an α1bAR antibody, showed a signal at 57 kDa, corresponding to the size of α1bAR (Fig. 3C). These bands were not seen in cells expressing D2DR alone or in the mock-transfected cells, and this result confirms that the FLAG-tagged α1bAR binds to anti-FLAG agarose beads. When α1bAR was immunoprecipitated using anti-FLAG agarose beads, and the Western blot was probed using an anti-HA antibody, a band at 50 kDa was seen in cells co-expressing the receptors, corresponding to the size of D2DR (Fig. 3D). Bands at 50 kDa were also seen in cells that were not transfected with α1bAR. This signal could be due to detection of the denatured antibody conjugated to the agarose beads, because the size of this band corresponds to the predicted weight of the antibody heavy chain component. It is

unlikely that these bands represent detection of D2DR in the mock-transfected group because there was no D2DR observed in the soluble lysate samples from these cells, and since the banding pattern was very similar in cells expressing D2DR alone, it is most likely that the signal at 50 kDa is due to detection of heavy chain in these cells as well. These results indicate formation of a stable complex between α1bAR and D2DR.

This result was confirmed using anti-HA agarose beads. Transfection was analyzed using soluble lysate samples from cells in each transfection group. When the blot was probed with an HA antibody, a band at 50 kDa was seen in the cells transfected with D2DR (Fig. 4A), and when the blot was probed with an antibody against α1bAR, a band at 57 kDa was seen in the cells transfected with α1bAR (Fig. 4B). A band at 57 kDa was also seen in the soluble lysate from the mock-transfected cells, indicating endogenous expression of α1bAR. For the immunoprecipitation experiments, it was confirmed that D2DR binds to anti-HA agarose beads when the Western blot of immunoprecipitate samples was probed with an HA antibody, and a band at 50 kDa was seen in the lane expressing D2DR (Fig. 4C). Faint bands at 50 kDa were also seen in the cells not expressing D2DR, but this is likely due to cross-reactivity of the HA antibody used to probe the blot with the HA antibody conjugated to the agarose beads. To assess physical interactions between α1bAR and D2DR, the Western blot of immunoprecipitate samples using anti-HA agarose beads was probed with an α1bAR antibody, and a faint band was seen at 57 kDa in cells co-expressing α1bAR and D2DR (Fig. 4D). Although this signal is not very robust, detection of a complex at a higher molecular weight also suggests physical interactions between α1bAR and D2DR. The results of complex-formation between α1bAR and D2DR using anti-FLAG agarose beads and anti-HA agarose beads were confirmed in triplicates.

α1aAR & D1DR

Soluble lysate samples from HEK293 cells were analyzed to confirm transfection in each group. When the Western blot was probed with an anti-α1aAR antibody, a band at 79 kDa, the predicted size of α1aAR, was seen in cells transfected with α1aAR (Fig. 5A). There appeared to be some non-specific binding of the α1aAR antibody, which is commonly seen using commercial α1AR antibodies (Jensen et al, 2009), but the band at 79 kDa was only seen in cells transfected with α1aAR. When the blot was probed with a D1DR antibody, a band at 50 kDa was seen in the cells transfected with D1DR (Fig. 5B). Using anti-FLAG agarose beads to immunoprecipitate α1aAR, a band was seen at 79 kDa in the immunoprecipitate samples from cells co-expressing α1aAR and D1DR (Fig. 5C). These bands were not seen in the mock-transfected cells or cells expressing D1DR alone, and this result confirms that α1aAR binds to the anti-FLAG agarose beads. When the blot was probed with a D1DR antibody, a band at 50 kDa was seen in the cells co-expressing the receptors (Fig. 5D). A faint band at 50 kDa was also seen in the cells expressing D1DR alone, and likely indicates detection of the heavy chain from the anti-FLAG antibody on the agarose beads. These results indicate physical interactions between α1aAR and D1DR.

Using anti-HA agarose beads to immunoprecipitate D1DR, evidence of physical interaction between α1aAR and D1DR was seen. When the Western blot containing soluble lysate samples was probed with a D1DR antibody, a band at 50 kDa, the predicted size of D1DR, was seen (Fig. 6A) and when the blot was probed with an α1aAR antibody, bands at 79 kDa were seen in the cells transfected with α1aAR (Fig. 6B). There again seemed to be non-specific binding of the α1aAR antibody, but the banding pattern appears transfection-dependent, indicating detection of α1aAR in the soluble lysate of cells transfected with α1aAR. In the immunoprecipitate samples, a

Western blot probed with a D1DR antibody showed a band at 50 kDa only in cells transfected with D1DR, indicating that D1DR binds to the anti-HA agarose beads (Fig. 6C). When D1DR was immunoprecipitated and the Western blot was probed with an α1aAR antibody, a band was seen at 79 kDa in the cells co-expressing the receptors (Fig. 6D). Higher molecular weight bands were also detected by the α1aAR antibody in the immunoprecipitate samples from cells co-expressing the receptors. Combined, these results indicate physical interactions between the receptors. The results of coimmunoprecipitation of α1aAR and D1DR using anti-FLAG agarose beads and anti-HA agarose beads were confirmed in triplicate experiments.

α1aAR & D2DR

To confirm transfection, soluble lysate samples from cells in each transfection group were analyzed. When the Western blot was probed with an α1aAR antibody, a faint band at 79 kDa was seen in the cells transfected with α1aAR (Fig. 7A). Due to the weakness of this signal, the higher molecular weight band indicating detection of oligomerized forms of α1aAR is a more reliable indicator of the presence of α1aAR in these cells. When the blot was probed with an antibody against the HA-tag on D2DR, bands were seen at 100 kDa in the soluble lysate from cells transfected with D2DR (Fig. 7B). This is the predicted size of the homodimer of D2DR, and is used as confirmation of transfection, due to the lack of detection of the D2DR monomer. In the immunoprecipitate samples, a band is seen at 79 kDa when anti-FLAG agarose beads are used to immunoprecipitate α1aAR, and an α1aAR antibody is used to probe the Western blot (Fig. 7C). No bands at 79 kDa are seen in the cells not expressing α1aAR, confirming that α1aAR binds to the anti-FLAG agarose beads. When α1aAR was immunoprecipitated with anti-FLAG agarose beads, it was seen that D2DR co-immunoprecipitates with it, and the Western blot shows a band at 50 kDa, the predicted

size of D2DR, when probed with an HA antibody (Fig. 7D). This provides evidence of formation of a stable complex between α1aAR and D2DR.

When anti-HA agarose beads were used to immunoprecipitate D2DR, these results were confirmed. D2DR and α1aAR were transfected into HEK293 cells, as seen by analysis of the soluble lysate samples. When an HA antibody was used to probe the Western blot of soluble lysate samples, a band at 100 kDa was seen, which is consistent with the size of the D2DR homodimer (Fig. 8A). Using an α1aAR antibody to probe the blot, α1aAR was detected in the soluble lysate of cells transfected with α1aAR, as indicated by the presence of α1aAR oligomers at high molecular weights in these cells (Fig. 8B). In the immunoprecipitation experiments using anti-HA agarose beads, D2DR was immunoprecipitated, as seen by the band at 50 kDa in the immunoprecipitate samples from cells expressing D2DR, when probed with an HA antibody (Fig. 8C). When D2DR was immunoprecipitated using anti-HA agarose beads, α1aAR was detected in the immunoprecipitate samples when the blot was probed with an α1aAR antibody. Although the blot showed a high amount of background and non-specific binding, α1aAR was detected in these samples by signals at a high molecular weight, which likely indicate detection of α1aAR oligomers (Fig. 8D). These results show that α1aAR and D2DR are capable of forming complexes, and the experiments were performed in triplicates.

Assessment of functional significance of complex formation between dopaminergic and adrenergic receptors

The surface luminescence assay was employed to measure changes in cell surface expression of α1bAR in HEK293 cells upon stimulation with phenylephrine, an α1AR agonist, and dopamine, a DR agonist, in the presence and absence of D1DR expression.

When α1bAR was co-expressed with D1DR, and phenylephrine was used to stimulate α1bAR, 27.76% of the receptors internalized, and a similar level of internalization (33.02%) was observed upon phenylephrine stimulation of cells expressing only α1bAR (Figure 9). When DA was used to stimulate D1DR, a 25.50% increase in surface expression of α1bAR was seen in cells co-expressing the receptors, but not in cells expressing α1bAR alone (Figure 9). These results were obtained from three replicates in each transfection and drug treatment group. However, this constitutes one trial of the assay, and these results must be replicated in order to assess statistical significance of the data.

DISCUSSION

The purpose of this study was to assess the mechanisms underlying the reported biochemical and behavioral interactions between α1ARs and D1DRs. Given previous data indicating colocalization of these receptors in rat PFC dendrites (Mitrano, 2010), along with the ability of some GPCRs to heterodimerize (Milligan 2006), it was hypothesized that α1ARs and D1DRs physically associate and form a novel signaling complex. Results from co-immunoprecipitation studies in HEK293 cells indicate that α1bARs and D1DRs are capable of forming complexes with molecular weights consistent with heterodimers in many cases. To our knowledge, this is the first evidence of heterodimer formation between any subtype of ARs and DRs, and α1bAR-D1DR heterodimers may contribute to reinstatement of cocaine seeking mediated by NE and DA in the PFC.

This interaction is not limited to α1bAR and D1DR, as several α1AR and DR combinations showed evidence of heterodimer formation. This can be attributed to the high similarity in protein sequence between the α1AR subtypes and the DR subtypes. α1aAR and α1bAR have 75% sequence homology, and the protein sequences of D1DR

and D2DR show 39-41% homology (Xhaard et al, 2006). Thus, various combinations of α1AR-DR heterodimers may mediate catecholamine signaling in diverse brain regions and cell types. Future experiments can be conducted to assess the ability of α1dARs to physically interact with D1DRs and D2DRs. A previous study found that D2DR could not increase α1dAR surface expression (Uberti et al, 2005), suggesting that this combination of α1AR and DR cannot form heterodimers, but this must to be tested directly via co-immunoprecipitation studies. When expressed alone, α1dAR is primarily localized intracellularly, making it more difficult to study these receptors (Hague et al, 2003). However, co-expression of α1dAR with certain other GPCRs such as α1bAR and β2AR has been shown to help α1dAR traffic to the cell surface (Hague et al, 2003; Uberti 2005).

Having shown evidence for heterodimerization between α1AR and DR subtypes in HEK293 cells, experiments verifying the formation of heterodimers between these receptors in cultured neurons and brain tissue can be performed. Because overexpression of transfected receptors in heterologous cells can sometime force complex formation that is not physiologically relevant, it will be important to assess whether the same interactions can occur in native cells and *in vivo*. Cell lysate samples obtained from neuronal cell cultures and brain tissue homogenate from rat PFC can be used in co-immunoprecipitation studies assessing heterodimerization between various subtypes of α1ARs and DRs. In addition, the use of more specific α1aAR and D2DR antibodies will provide clearer evidence of heterodimerization among these receptors.

It was also shown that receptor trafficking was altered by co-expression of α1bAR and D1DR. When agonized with phenylephrine, α1bAR internalized to a similar degree whether expressed alone or in combination with D1DR. In the latter condition,

we cannot be certain whether we were measuring the internalization of α1bAR-D1DR heterodimers or monomeric or homodimeric α1bARs. Although incubation with DA had no effect on α1bAR surface expression in cells expressing α1bAR alone, it increased cell surface expression of α1bAR in cells co-expressing α1bAR and D1DR. While still preliminary, this result suggests that ligand-induced activation of D1DR alters trafficking of α1bAR when the receptors are co-expressed. There are several possible mechanisms that could account for this observation of increased cell surface expression of α1bAR when co-expressed with D1DR and stimulated with DA. For example, monomeric D1DR at the cell surface may have been internalized upon stimulation by DA, and through a rapid-recycling mechanism, internalized D1DR may have bound intracellular α1bAR and been trafficked to the membrane as a heterodimer, thus increasing the surface expression of α1bAR. However, additional experiments, such as time-lapse confocal microscopy, are necessary to determine the cause of the increased surface expression of α1bAR upon D1DR stimulation. In conjunction with a previous study reporting that α1AR stimulation causes accelerated resensitization of D1DR (Trovero et al, 1994), it seems likely that that this interaction occurs in the reverse scenario as well (i.e. stimulation of α1AR increases surface expression of D1DR). The percent change in cell surface expression from baseline should also be analyzed for D1DR, alone and when co-expressed with α1bAR, and stimulated with selective α1AR and D1DR agonists, such as phenylephrine and SKF81297, respectively. These results will provide a comprehensive view of cell surface expression patterns and trafficking of the α1bAR-D1DR heterodimer in HEK293 cells. In addition, because putative heterodimers were seen between α1bAR and D2DR, α1aAR and D1DR, and between α1aAR and D2DR, receptor internalization and trafficking should also be studied for these combinations of receptors.

Other aspects of GPCR heterodimer functionality can be further explored as an extension of this research. Studies analyzing receptor signaling can test the effects of agonist stimulation on downstream signal transduction. Any studies involving measurement of signaling products will rely on knowledge of how the receptor's affinity for the ligand might be altered when a heterodimer is formed, which provides another avenue for future directions of this research. Subsequently, experiments measuring levels of cyclic adenosine monophosphate (cAMP), a product of DR signaling, in cells co-expressing various subtypes of α1AR and DR, can be used as an indicator of functional activity of DR. Inositol phosphate (InsP) is a downstream pathway product of α1AR signaling, and measurement of InsP levels in cells co-expressing α1AR and DR subtypes can be used to assess functional activity of α1AR subtypes upon stimulation with phenylephrine.

Future directions of this research on α1bAR and D1DR internalization include analyzing receptor internalization after administration of D1DR and α1bAR agonists *in vivo*. In brain tissue from rats treated with these drugs, double-labeling immunohistochemistry can be performed, and cell surface expression of α1bAR and D1DR can be measured in neuronal elements containing expression of only one receptor, compared to when the receptors are co-expressed. This will provide more salient information regarding the functionality of α1bAR-D1DR heterodimers *in vivo*.

In addition to the proposed additional experiments, the results of this research may facilitate our understanding of adrenergic and dopaminergic receptor interactions in the PFC, and provide additional insight into the mechanisms by which these systems interact with one another in this brain region. The implications of α1AR interactions with D1DRs in the reinstatement of cocaine seeking can be explored further, and these

results may lead to investigation into novel therapeutic targets for drug addiction and reinstatement.

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TABLES AND FIGURES

Antigen	Immunogen	Manufacturer Data	Dilution Used
Alpha-1a adrenergic receptor	Adrenergic, alpha-1A-, receptor recombinant protein epitope signature tag (PrEST)	Sigma-Aldrich	1:500
Alpha-1b adrenergic receptor	15 amino acid peptide from the C terminal residues of human alpha 1b Adrenergic receptor	Abcam	1:20000
D1 dopaminergic receptor	Recombinant fusion protein containing the C-terminal 97 amino acid of human D_1 dopamine receptor	Sigma-Aldrich	1:2000
HA epitope	HA peptide sequence (YPYDVPDYA) derived from the influenza hemagglutinin protein.	Roche Applied Science	1:1000
Anti-FLAG M2-Peroxidase	FLAG epitope	Sigma-Aldrich	1:1000
FLAG agarose antibody	FLAG epitope	Sigma-Aldrich	N/A
HA agarose antibody	Synthetic peptide corresponding to amino acid residues 98-106 of human influenza virus hemagglutinin	Sigma-Aldrich	N/A

Table 1. Immunological reagents used in co-immunoprecipitation, Western blots, and surface luminescence assay

Receptor Type	Size (kDa)
D1-dopaminergic receptor	50
D2-dopaminergic receptor	50
α1a-adrenergic receptor	79
α1b-adrenergic receptor	57

Table 2. Molecular weights of the various dopaminergic and adrenergic receptor subtypes most robustly detected by the primary antibodies (obtained from antibody product information)

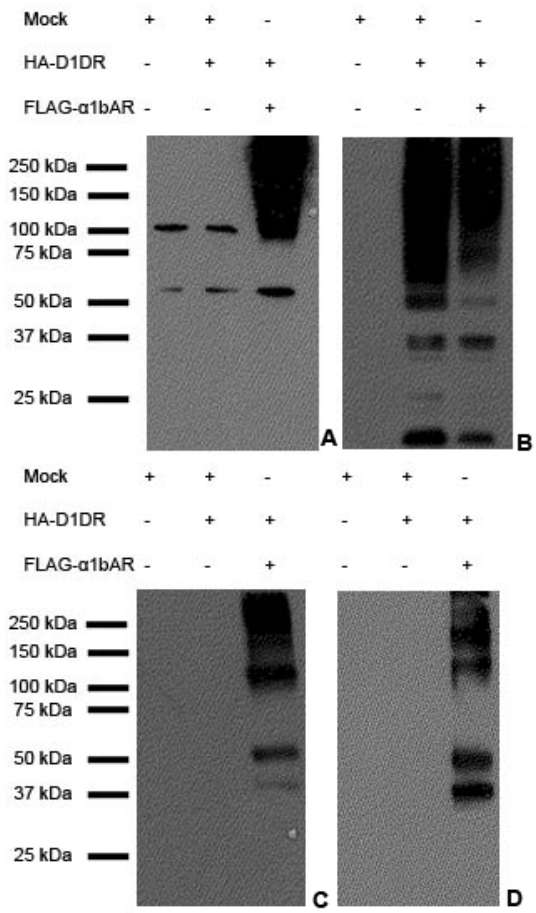

Figure 1. (A) Soluble lysate samples probed with an α1bAR antibody (B) Soluble lysate samples probed with a D1DR antibody (C) Immunoprecipitate samples from anti-FLAG agarose beads probed with an α1bAR antibody (D) Immunoprecipitate samples from anti-FLAG agarose beads probed with a D1DR antibody

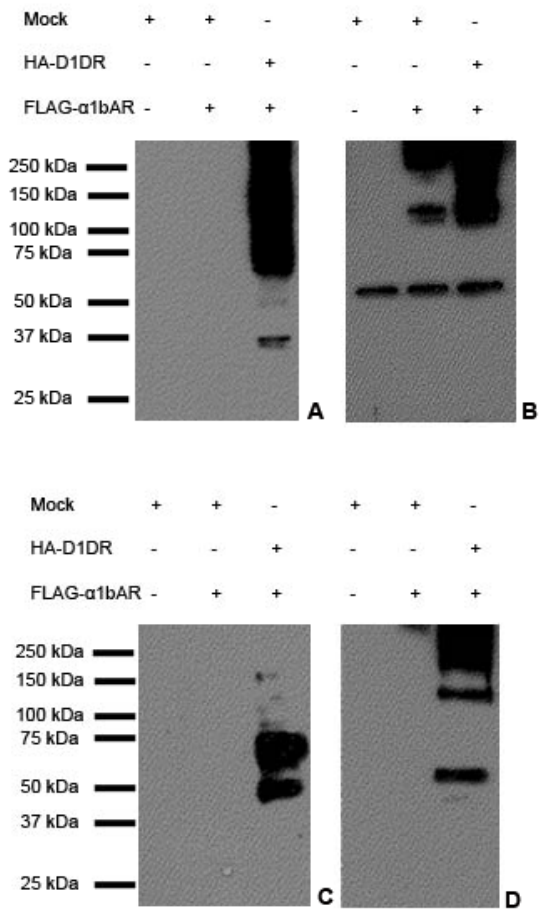

Figure 2. (A) Soluble lysate samples probed with a D1DR antibody (B) Soluble lysate samples probed with an α1bAR antibody (C) Immunoprecipitate samples from anti-HA agarose beads probed with a D1DR antibody (D) Immunoprecipitate samples from anti-HA agarose beads probed with an α1bAR antibody

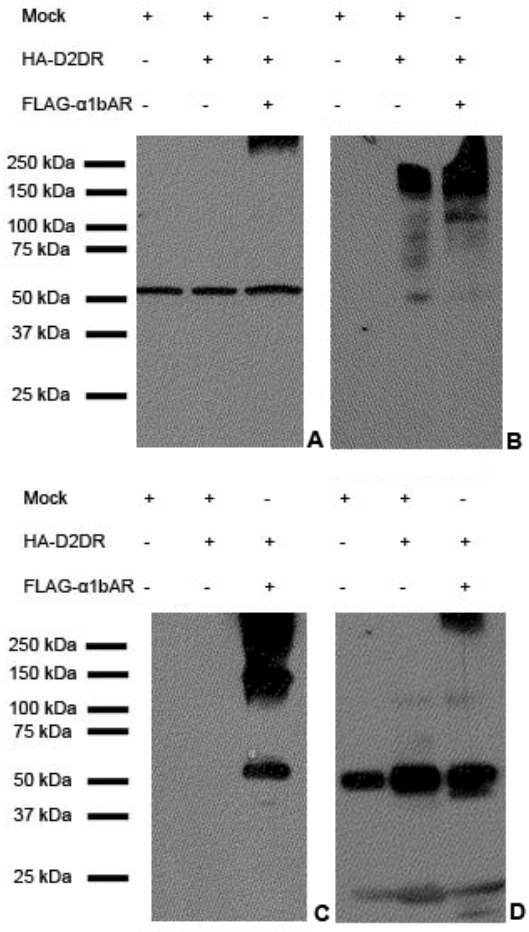

Figure 3. (A) Soluble lysate samples probed with an α1bAR antibody (B) Soluble lysate samples probed with an HA antibody (C) Immunoprecipitate samples from anti-FLAG agarose beads probed with an α1bAR antibody (D) Immunoprecipitate samples from anti-FLAG agarose beads probed with an HA antibody

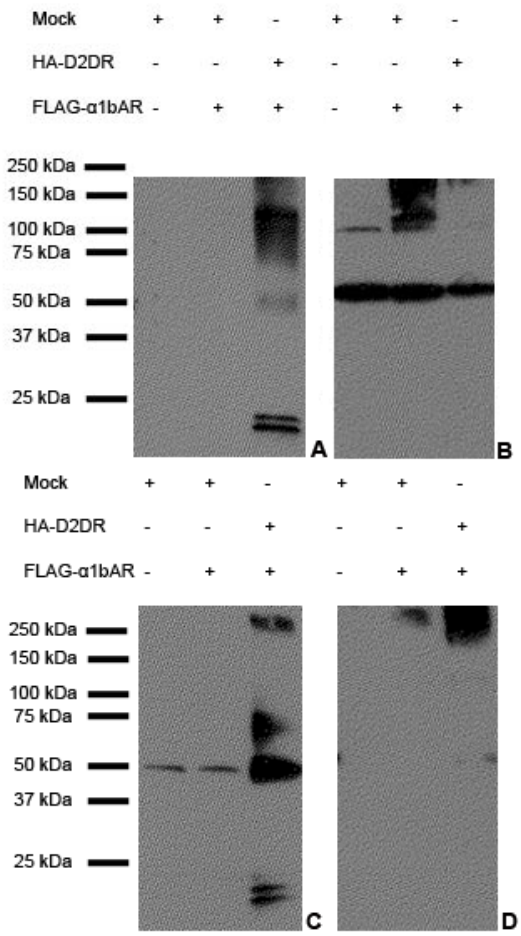

Figure 4. (A) Soluble lysate samples probed with an HA antibody (B) Soluble lysate samples probed with an α1bAR antibody (C) Immunoprecipitate samples from anti-HA agarose beads probed with an HA antibody (D) Immunoprecipitate samples from anti-HA agarose beads probed with an α1bAR antibody

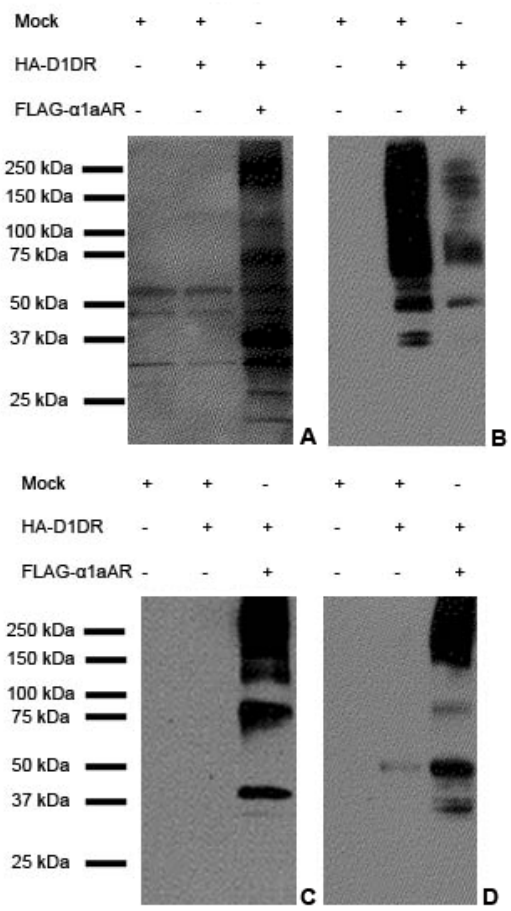

Figure 5. (A) Soluble lysate samples probed with an α1aAR antibody (B) Soluble lysate samples probed with a D1DR antibody (C) Immunoprecipitate samples from anti-FLAG agarose beads probed with an α1aAR antibody (D) Immunoprecipitate samples from anti-FLAG agarose beads probed with a D1DR antibody

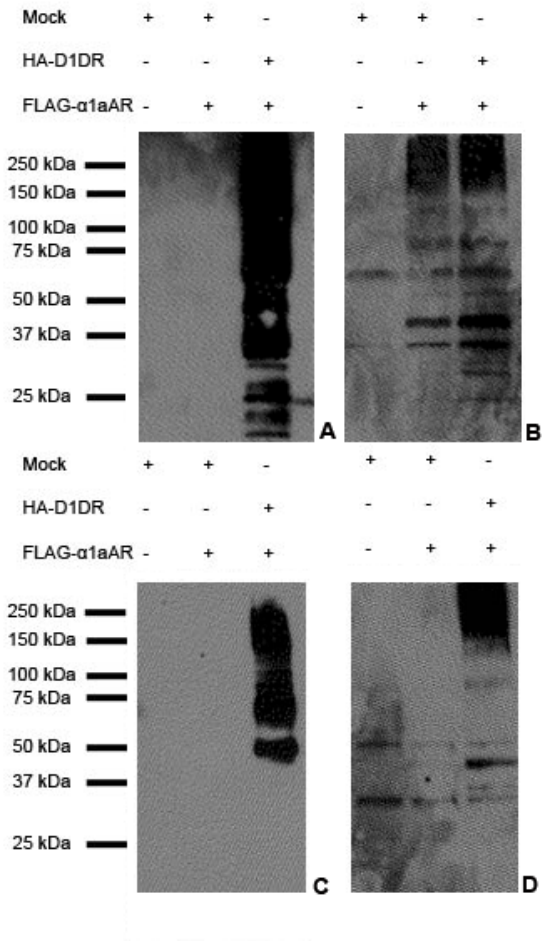

Figure 6. (A) Soluble lysate samples probed with a D1DR antibody (B) Soluble lysate samples probed with an α1aAR antibody (C) Immunoprecipitate samples from anti-HA agarose beads probed with a D1DR antibody (D) Immunoprecipitate samples from anti-HA agarose beads probed with an α1aAR antibody

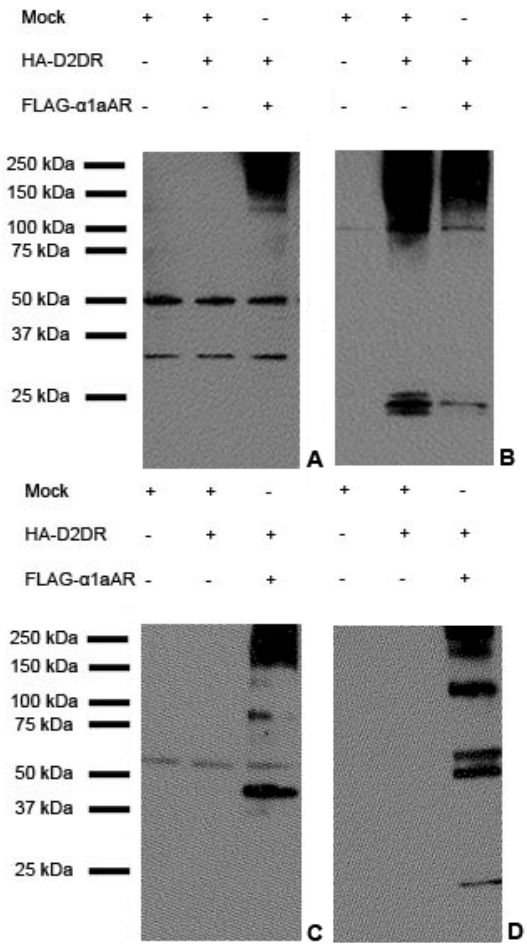

Figure 7. (A) Soluble lysate samples probed with an α1aAR antibody (B) Soluble lysate samples probed with an HA antibody (C) Immunoprecipitate samples from anti-FLAG agarose beads probed with an α1aAR antibody (D) Immunoprecipitate samples from anti-FLAG agarose beads probed with an HA antibody

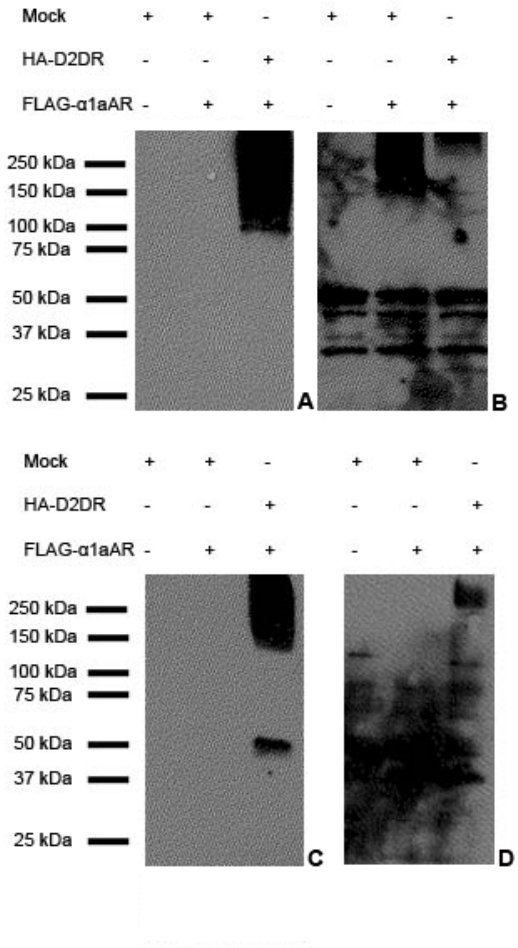

Figure 8. (A) Soluble lysate samples probed with an HA antibody (B) Soluble lysate samples probed with an α1aAR antibody (C) Immunoprecipitate samples from anti-HA agarose beads probed with an HA antibody (D) Immunoprecipitate samples from anti-HA agarose beads probed with an α1aAR antibody

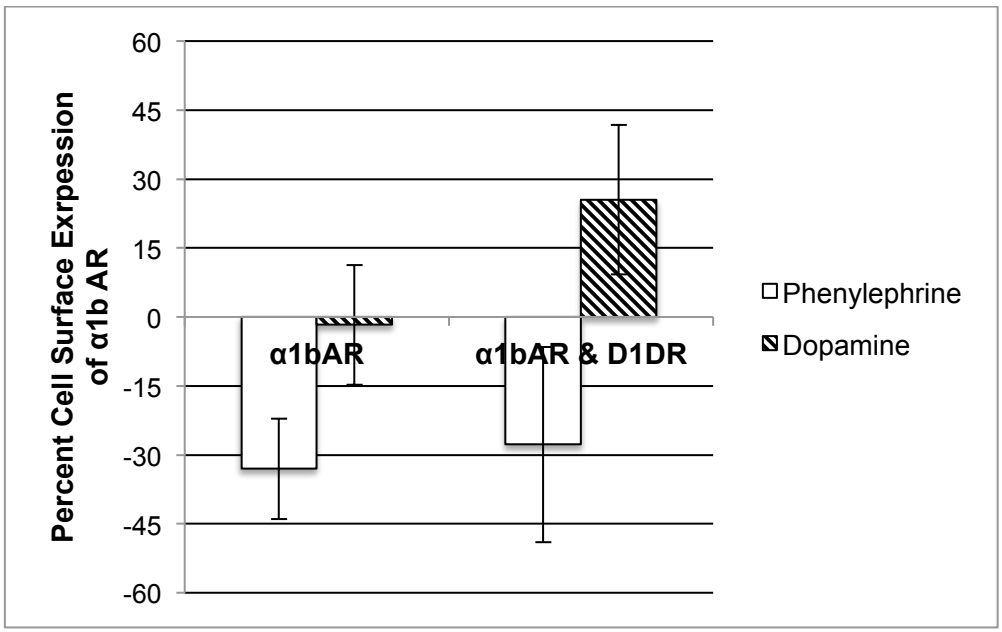

Figure 9. Percent cell surface expression of α1bAR from baseline (PBS w/ Ca^{+2}) in HEK293 cells expressing α1bAR alone or α1bAR and D1DR, after treatment with phenylephrine or dopamine